

UNCLASSIFIED

AD 418716

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

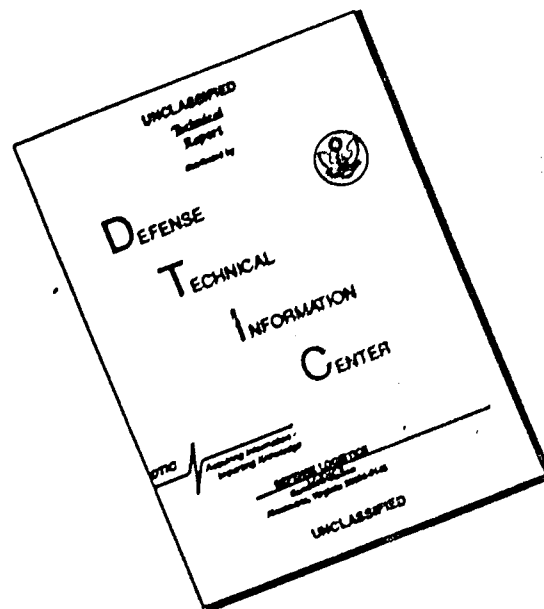
CAMERON STATION, ALEXANDRIA, VIRGINIA



UNCLASSIFIED

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

418716

INFLUENCE OF COLD ON HOST-PARASITE INTERACTIONS

INFLUENCE
of
COLD
on
HOST-PARASITE
INTERACTIONS

VIERECK

BY DDC

AS AD 10.

Editor

ELEANOR G. VIERECK

64-4

CATALOGUE
AS AD 10

418716

DDC
JUL 7 1963
JISIA D

ARCTIC AEROMEDICAL LABORATORY
FORT WAINWRIGHT
ALASKA
1963

**INFLUENCE OF COLD
ON HOST-PARASITE INTERACTIONS**

PART I

Editor

ELEANOR G. VIERECK

ARCTIC AEROMEDICAL LABORATORY
FORT WAINWRIGHT
ALASKA

1963

PROCEEDINGS

SYMPOSIA ON ARCTIC BIOLOGY AND MEDICINE

III. INFLUENCE OF COLD ON HOST-PARASITE INTERACTIONS

Symposium held August 28, 29, 30, 1962 at the
Arctic Aeromedical Laboratory
Fort Wainwright, Alaska

Symposium Organizer and Chairman

L. Joe Berry
Department of Biology, Bryn Mawr College

Editor

Eleanor G. Viereck
Research Physiologist, Arctic Aeromedical Laboratory

Symposium held under the auspices of the
Geophysical Institute
University of Alaska
College, Alaska

1963

TABLE OF CONTENTS

Part I.

1. Introduction to the Symposium, Influence of
Cold on Host-Parasite Interactions, L. Joe Berry 1
2. Difficulties in Epidemiological Studies of the
Relationship of Cold to Human Infections,
Robert I. McClaughry 11
3. Aspects of Arctic Epidemiology, Frank L. Babbott, Jr. 25
4. The Ecology of Enteroviruses in Alaska,
Karl R. Reinhard 47
5. Opening Remarks on Problems of Immunization
in Stressed Animals, Dan H. Campbell 81
6. Environmental Extremes and Endocrine Relationships
in Antibody Formation, Ignatius L. Trapani 89
7. Qualitative and Quantitative Aspects of the
Immune Response under Conditions of Cold
Exposure, William T. Northey 109
8. Influence of Hypothermia on the Action of
Bacterial Toxins, G. Tunevall and T. Lindner 135

Part II.

9. Effect of Low Ambient Temperatures on Specific
and Nonspecific Resistance, Fred Miya, Stanley
Marcus, and LeGrande J. Phelps 155
10. Virulence as a Factor in Host Response to
Bacterial Infection at Low Environmental
Temperature, Joseph J. Previte and L. Joe Berry 215

11. Endogeneous and Experimental Peritonitis and
Bacteraemia in Hypothermic Mice, G. Tunevall
and T. Lindner 239

12. The Role of Low Environmental Temperatures
in Predisposing Mice to Secondary Bacterial
Infection, Gennaro J. Miraglia and L. Joe Berry 271

Part III.

13. Cold and Colds, Sir Christopher Andrewes 301

14. Effect of Environmental Temperature on
Viral Infection, Duard L. Walker, M. D. 319

15. The Influence of Cold on Virus Infectivity,
Dr. T. G. Metcalf 343

16. Suppressive Effect of Low Environmental
Temperature on Viral Infection in Bats,
S. Edward Sulkin and Rae Allen 369

17. Microbiological Aspects of Hibernation in
Ground Squirrels, J. Schmidt 399

18. Cold Therapy in Bacteremic Shock, Emil Blair 419

19. Summary of the Symposium, Walter J. Nungester 447

DISCUSSANTS

HORACE F. DRURY, Arctic Aeromedical Laboratory,
Fort Wainwright, Alaska.

BOB HUNTLEY, Arctic Health Research Center, Anchorage,
Alaska.

R. B. MITCHELL, School of Aerospace Medicine, Brooks
Air Force Base, Texas.

JOHN A. MONCREIF, Brooke Army Medical Center,
Fort Sam Houston, Texas.

CHARLES T. MARROW, Fairbanks Medical and Surgical
Clinic, Fairbanks, Alaska.

This publication has been released to the Office of
Technical Services, U. S. Department of Commerce,
Washington, 25, D. C., for sale to the general public.

INTRODUCTION TO THE SYMPOSIUM
INFLUENCE OF COLD ON HOST-PARASITE INTERACTIONS

L. Joe Berry

Department of Biology
Bryn Mawr College
Bryn Mawr, Pennsylvania

The purpose of the Symposium is familiar to all of you, but permit me the privilege of stating it in my own way. It is our task in the two and one-half days ahead to look critically at an area that is part science, part superstition, and part unknown, with the view of determining how much time, effort, and money should be spent in its study. We must ask now and throughout the proceedings whether cold is an important factor in modifying or altering host-parasite interaction. We have been brought here by a branch of the Armed Forces of the United States because the medical authorities of that branch charged with the responsibilities of safe-guarding the health of military and civilian personnel, who are, for the sake of the security of our country, forced to live in hostile environments, need to have an answer. They are looking to us for help, and if it is within our wisdom to provide, I know we will. There is before us, therefore, a very real and practical problem which is our primary concern; but there is also the challenge of pure science. Medical and biological science from its earliest history has been preoccupied and inquisitive about the role the environment plays in the behavior and response of living organisms. I do not know to what extent the phases of the moon exert an influence at the time of planting or cultivating, or harvesting the yield and value of crops, but I have heard in my youth from many firm and dogmatic assertions by practicing farmers that they do. I also recall with some nostalgia my mother, my great aunts, and indeed, our faithful country doctor warning against wet feet, cold on the back of the neck, night air cooling off too fast from a good sweat and the dire consequences (all of an infectious nature) that would otherwise result. I remember with affection my mother's wish to protect me from the hostile

BERRY

rigors of a winter in the far north of Texas. Each October I went into long white underwear only to emerge in late March or early April when the air warmed to a comparatively safe 80° F to 90° F. Many of these notions are firmly implanted in the minds of millions of people more from reiteration than by demonstration, to borrow a phrase from a friend (Schneider, 1946). But as is often the case, where conviction is firm, there is often more than a superstitious quicksand to give it support. We can now begin by examining some of the bases for this support, and, I hope, finding what there is of merit, what there is that is formless and amorphous, and where, if anywhere, new work is needed to give form to our knowledge.

In undertaking the responsibility of organizing this Symposium, it seemed to me that attention should first be directed to an analysis of past experience of Armed Forces in an arctic environment. During World War II and certainly in Korea, large bodies of men were forced to live and fight at cruelly low temperatures. This was particularly true on the Russian front (for which no direct knowledge is readily accessible), but knowledge is available to an adequate degree around the Chosin reservoir in Korea. Casualties from frostbite were acknowledged, but the extent to which trained and seasoned troops were made more susceptible to microbial disease may never be known. It is to obtain as much information as possible on these points that Dr. McClaughry was asked to contribute his paper. This was an unusually difficult and complex assignment, and we are grateful to him for his willingness to undertake it.

The epidemiology of the Arctic poses problems distinct from those normally encountered. This fact has been recognized by qualified investigators, and hence the presence of Dr. Babbott and Dr. Reinhard. These men have each spent extended periods of time studying the unusual facets that make arctic epidemiology so challenging. Their experiences, it must be borne in mind, deal primarily with indigenous populations living under conditions potentially unrelated to those that might arise in a military emergency. Perhaps in the discussion it will be possible to obtain estimates of what might be expected under other circumstances and whether there are areas where further work is needed. It is

INTRODUCTION

self-evident that knowledge gained from studies of native populations selected genetically through the centuries for life in the Arctic might conceivably have little applicability to the behavior of a population of greater size and concentration suddenly moved into this environment.

Cold is a stress. How much it results in a unique response by the body and the degree to which it resembles other stresses must be left to the physiologists for elucidation. They have already established that acclimatization to cold entails greater heat production through an elevation in metabolic rate as judged by increased oxygen consumption. This is believed to be mediated through the endocrines in a highly complex, and as yet incompletely understood manner (Hart, 1958). These facts have pertinence for anyone interested in host-parasite interactions because of the well documented contributions host metabolism makes to determining health or illness from an infectious agent. Dubos' book, The Biochemical Determinants of Microbial Disease, (1954), is a milestone, and since its appearance, numerous publications have expanded the literature.

Mammals exposed to cold can be expected to maintain normothermia unless there is excessive heat loss. Hypothermia is probably the consequence of too little heat production and too great heat loss. The investigator must constantly be prepared to discriminate, therefore, between effects in normothermic cold exposed animals and those found in hypothermic cold exposed animals. The literature contains all too many examples of work in which these distinctions were not made.

The major question confronting us is: Does cold exposure influence detrimentally man's ability to combat infectious diseases? This question raises many more. What diseases? What men? How much cold and for how long? It is obviously difficult to answer these questions, particularly with man as the experimental subject, except for the most unusual circumstances such as those Sir Christopher Andrewes has used so imaginatively in his studies of the common cold. To a highly limited extent, it might be possible to utilize human volunteers for experimental infection with a few diseases against which highly therapeutic agents are available. So far as I know,

BERRY

nothing of the kind has been attempted. Should it be done? Well might we provide the answer.

The uncertainties, the difficulties, and indeed the impossibilities of scientific experimentation with human beings in most research with infectious diseases forces the use of laboratory animals. There is ample precedence in the area of cold since a century ago when Pasteur reported his classical work with anthrax in chickens. The birds were resistant until chilled by a cold rain (Pasteur et al., 1878). In the intervening years the literature is adequate to prove that Pasteur's observations were not isolated findings. Other animals may or may not respond in modified form to infectious challenge, depending upon a number of variables, but there are sufficient examples to leave no room to doubt that host-parasite interaction is a plastic phenomenon under the pressure of certain environmental changes.

Confrontment with the established fact still leaves an enormous area for elaboration. When animals are known to become more susceptible to infection, there is always the question of why. It is axiomatic by now to offer certain tentative explanations. It can be said, for example, that the defenses of the host have been weakened. These include the humoral defense, the cellular defense, a weakening of the barriers to invasion, plus various combinations of them all. Even if there is evidence for change in any one of the defenses, it is still necessary to seek out the basis for the change. It is also possible that virulence of the pathogen is enhanced. Without attempting to define virulence, agreement hopefully is assumed that it comprises many facets, any one of which may become primary in a particular situation. Again, it is desirable to understand the nature of the virulence change whenever it plays a role. There are, in addition, somewhat more elusive concepts that can be reasonably offered to explain an elevation in host susceptibility. The in vivo environment on which pathogen proliferation depends, be it intracellular, extracellular, or both, can conceivably be enriched. There is less direct evidence for this than one would wish, especially for the bacteria, but virus multiplication is known to be intimately linked with the metabolic vigor of the parasitized cell. By contrast, the host might be put in the position of heightened sensitivity to the toxic manifestations of the disease state.

INTRODUCTION

The success of any Symposium usually hinges on the participation of a few key individuals. One such person, in my judgment, whose early commitment as a participant undoubtedly influenced the acceptance of others, is Dr. Dan Campbell. He will lead the session this afternoon on the influence of cold on the immune response. Rather than give the impression of attempting to anticipate his remarks (which I would never presume to do), I am going to comment only briefly on this topic. Immunologists have refined their techniques to such a degree that they are the envy of many research biologists. Indeed, their methods have been applied wherever possible to problems fundamentally alien to those classically within the scope of their specialty. Immunology is quantitative biology at its best, and may possibly be considered the parent of molecular biology. To determine with a high degree of precision the level of response to antigenic stimulation does not necessarily make evident, however, the reason why one group of animals reacts differently from another. We can expect to learn more about these phenomena from Drs. I. L. Trapani and William Northey, who have been concentrating in this area of work for some years. It is a great pleasure to welcome not only them, but also one of two members of the group who make this Symposium international. Dr. Gosta Tunevall will speak first on the effects of hypothermia on the response of mice to bacterial toxins. This is one of four papers (two from Dr. Tunevall) dealing specifically with animals at reduced body temperature as it influences host-parasite interaction. The first is concerned with the important problem of how hypothermia alters the physiological or pharmacological properties of a soluble toxin. These bacterial products must exert their primary action at the enzymic level. If hypothermia reduces metabolic rate, as it must since the velocity of all chemical reactions is lowered by a drop in temperature, then there should be a less acute action of toxin in animals at reduced body temperature. As logical as this may seem, there are also other alternative effects that would make this untrue. Hypothermia, as it becomes more and more severe, affects different organs such that their activities do not change in parallel (Adolph, 1959). Physiological distortion occurs, compared to the integrated normal state, such that some functions cease at a temperature where others continue. Breathing may stop at about 15° C, but the animal can survive and recover if artificial respiration is administered. Cessation of heart beat then becomes a cause of death in animals so maintained and

BERRY

further cooled. These facts have relevance only to serve as warnings against any attempt to generalize about the hypothermic influence on host-parasite interaction. The specific body temperature and its duration must be specified in reporting results, and conclusions can never be presumed generally applicable unless data are available to prove it.

The two sessions tomorrow will attempt to answer the question: Does cold predispose experimental animals (and man) to infectious disease, and if so, what diseases and under what conditions? We are unusually fortunate in having two of the world's authorities on infectious diseases to serve as moderators. I refer, of course, to Dr. Walter Nungester and to Sir Christopher Andrewes. Dr. Nungester has specialized primarily as a bacteriologist and Sir Christopher as a virologist. Their names must certainly be included in the special list of experts whose willingness to participate contributed so much in attracting to the Symposium such an illustrious group of participants. We are delighted to welcome and express our deepest gratitude to Drs. Miya, Previte, and Miraglia for the reports they are to give on work with bacterial diseases. We are equally fortunate in having such distinguished virologists interested in this area of research. I refer, of course, to the Drs. Walker, Metcalf and Sulkin, and to perhaps the most versatile of us all, Dr. Marcus, who is at home with both bacterial and viral diseases as well as with immunology.

A warning has been sounded against the pitfalls of generalizing from experiments with hypothermic animals unless sufficient knowledge justifies it. A similar warning must be issued in regard to cold exposure and change in susceptibility to infection. Differences in interpretation of results, differences in experimental design, and differences in findings are to be not only anticipated but desired. It is in this way that breadth of understanding is best acquired. But let us reflect a moment on some of the obvious difficulties in this type of work. At what temperature are the animals exposed? "Cold", according to the literature, can be anything from 15° C to 20° C down to refrigerator temperatures, about 5° C, or deep freeze, -10° C to -20° C, or it can be an arctic blizzard of -45° C. How long are the animals exposed? This can range all the way from continuous exposure, beginning with challenge, to an infinite varia-

INTRODUCTION

tion of intermittent exposures before or after infection. Are the animals cold acclimatized? One must ask how acclimatization is determined. Does it merely mean exposure for some time prior to infection, or is there some measurement employed that proves the animals are cold tolerant? Of greatest importance, in my judgment, are detailed descriptions of experimental conditions of the exposure to cold. Are the animals housed separately or in groups? Is bedding material provided? If they are singly housed, how much space is available? What is the velocity of air movement around the animals? Is relative humidity controlled, and if so, at what saturation? Are food and water continuously available? I confess as much ignorance about these matters as most anyone, yet a few preliminary experiments have clearly indicated that these factors cannot be ignored. Another consideration is illumination. Continuous dark, continuous light, or intermittent and erratic light are all to be avoided. Physiologists concerned with biological clocks or with periodicity in higher organisms have established beyond reasonable doubt that carbohydrate reserves, body temperature, endocrine secretion, eosinophile counts, and many others are all subject to highly significant variations from one time of day to another (Halberg, 1960). Some of these changes may be correlated with light, while others may show free-running periods not directly associated with detectable environmental phenomena. Space in which the animal is housed, cubic volume as well as area, may contribute in unsuspected degree to certain responses under investigation. The exigencies of space travel is making this an important object of study. Research in infectious diseases may be able to add something of significance to this work.

As moderator for our final session, it is a particular pleasure for me to welcome a very close friend of many years. We took several graduate courses together, our doctoral research was carried out in laboratories opening into the same basement corridor, we shared our frustrations and successes with one another, and we walked across the platform the same hot June night in Texas more years ago than either of us cares to admit to receive our Ph. D. degrees. Dr. R. B. Mitchell has been the author of numerous scientific papers, and is now continuing his service to science through primarily administrative channels as Chief of the Department of Medical Sciences, School of Aerospace Medicine.

BERRY

The final session contains the only paper on hibernation that is to be presented. Several participants suggested that someone concerned with this subject be invited, but most investigators are interested in the physiology of the hibernating animal. Little attention has been paid, however, to the fascinating possibilities such animals provide for the study of host-parasite interactions. Mr. Schmidt, who spent several years at the Arctic Laboratory, is a pioneer in this work and will, I am sure, be welcomed back to Alaska by his many friends.

The program is to be concluded by two men, Drs. Emil Blair and Colonel John Moncrief, who are really on the front line of the fight for knowledge about cold and infectious diseases. They have successfully used hypothermia as a therapeutic tool primarily against Gram negative infections in human beings. They are also applying their clinical experience to the design of animal experiments capable of elucidating some of the mechanisms involved in patient recovery. The significance of this work is evident, and we will be pleased to hear of the progress made in this applied field.

Before proceeding with the program, I want on behalf of us all to express our appreciation to the Arctic Laboratory for making this Symposium possible; to Mr. Robert Becker for the detailed arrangements he has made for our comfort and relaxation; to Mr. Alfred George of the University of Alaska for his travel arrangements; and to Dr. Eleanor Viereck for her editorial assistance, we voice our thanks. We are grateful to Major Sproul, Commandant of the Laboratory, for the hospitable accommodations he has provided for these sessions.

As you are aware, Colonel John D. Fulton is not here. This Symposium is his dream, and I hope it proves to be all he expected. He asked me to undertake its organization and has given at all times his fullest cooperation. If there are any omissions or deficiencies in the organization of the scientific program, they are mine and not his.

INTRODUCTION

LITERATURE CITED

1. Adolf, E. F. 1959. Zones and stages of hypothermia. *Ann. N. Y. Acad. Sci.* 80: 288-290.
2. Dubos, R. J. 1954. Biochemical determinants of microbial disease. Harvard Univ. Press, Cambridge. 152 p.
3. Halberg, F. 1960. Temporal coordination of physiologic function. *Cold Spring Harbor Symp. Quant. Biol.* 25: 289-310.
4. Hart, J. S. 1958. Metabolic alterations during chronic exposure to cold. *Fed. Proc.* 17: 1045.
5. Pasteur, L., J. F. Joubert, and C. Chamberland. 1878. La theorie des germes et ses applications à la médecine et à la chirurgie. *Bull. Acad. de Med., Paris, 2nd Series.* 7: 432-447.
6. Schneider, H. 1945. Nutrition and resistance to infection: the strategic situation. *Vitamins and Hormones* 4: 35-70.

DIFFICULTIES IN EPIDEMIOLOGICAL STUDIES
OF THE RELATIONSHIP OF COLD TO HUMAN
INFECTIONS

Robert I. McClaughry

Department of Medicine and Surgery
Veterans Administration
Washington 25, D. C.

ABSTRACT

This report deals with the difficulties experienced in an attempt to use Veterans Administration hospital records to determine the influence of cold on infectious disease. Various means were explored to find if exposure to cold of the members of the Armed Forces of the United States engaged in the Korean War influenced the occurrence and course of infectious diseases.

Scientific activity has risen exponentially in recent years. One result of this phenomenon has been the accumulation of a very large body of data. Dissemination of this information poses a major problem, which has been met in part by more scientific meetings and journals. Strange as it seems at times to a scientist trying to find time to get into his laboratory, data has been obtained faster than outlets for its reporting have developed. In absolute terms, there is no dearth of material to be discussed in scientific circles. These events have made the ever difficult and unpopular task of presenting negative results even more problematical. After all too lightly accepting exactly this assignment at this symposium, the full weight of what I had undertaken descended upon me. Justifying a negative report to this illustrious group posed a problem of no mean proportions.

I request your indulgence, therefore, for a brief description of the reasons for my appearance here today. It all dates back to the early summer of 1959, when the Chairman of the Division of Medical Sciences of the National Academy of Sciences - National Research

MC CLAUGHRY

Council asked me to direct a survey of the medical research program of the Veterans Administration. In short order, I became acquainted with a strong opinion held by the then Administrator of Veterans Affairs, Mr. Sumner G. Whittier. He indicated that on the one hand there were the many Veterans Administration medical records, and on the other hand, there was the booming technology of electronic data processing. Now, to continue the paraphrase, if only a marriage of the two could be effected, surely answers to most important medical problems would be found.

The committee of medical scientists involved in the NRC study reached more circumspect conclusions from evaluating all the evidence available to them. Indeed, the final statement in the section on the use of computers in their report bears quotation in this context. "It is also to be noted that only very limited use can be expected of the medical records now in existence for retrospective studies, and that the collection of data by designing experiments for the use of computers should be the rule."

Despite this forewarning, which should certainly have been adequate, I fell into the trap of attempting a retrospective record study. In my defense, I can only say that the possibility of association of exposure to cold during the Korean campaign with differences in the incidence or course of infections seemed straightforward enough to merit a try by this method. I was also somewhat influenced by the consideration that such environmental influences on human infection have received relatively little attention, despite their importance.

The burden of my message is really very simple. It was completely impossible to establish from the records any group who were known to have been exposed to cold, or conversely to find a group similar except for such exposure. This information was simply not recorded. Furthermore, inference of the probability of exposure to cold by identifying the military organization to which an individual belonged proved fruitless.

Since I am nearly as sensitive as was the fabled jackass which was struck a hard blow on the head with a singletree to get its attention, I wistfully gave up the record study at this point. Optimist that I am, though, I must add that the prospect appears better for

DIFFICULTIES OF EPIDEMIOLOGICAL STUDIES

the success of a pre-planned epidemiological study of this question. From the perusal of medical records, it appears that a military population on maneuvers or in combat in a cold climate could be used for such investigations. It would be necessary to obtain definite information about the kind and degree of environmental exposure. Documentation of the diagnosis and treatment of various infections is now fairly standardized.

It is enough to indicate that I failed in the first approach to find a relation between exposure to cold and human infection, and that the information which was available may favor a different approach. To extend myself further would jeopardize my inherently unstable position by presuming to tell someone how his research should be done. For the problem passes from the range of studies possible with Veterans Administration records into the interest of those engaged in field studies.

Now, having spoken for about five of the forty minutes that the program allocated to this topic, I shall make my only significant contribution to this symposium by sitting down and making more time available for discussion of the substantive papers.

MC CLAUGHRY

DISCUSSION

MONCRIEF: I want to echo Dr. McClaughry's comments about retrospective studies, particularly clinical situations. They really have very little value because of the absence of the data that you are looking for in the clinical records. We have, I feel, probably one of the most exhaustive sources on clinical material and data collections anywhere in the country because we have a relatively small unit and a large number of personnel. Although a patient has been in the hospital two months and may have a clinical summary the size of a small telephone book, it still does not have the data in it we want in the future; and retrospective study is only valuable if the data that is contained in your records is positive; then it may be of some value. If it is negative, it may be that the doctor who made up the summary just left it out, or he may have observed it but did not put it down in the records, so the only thing you can say with retrospective study is whether something occurred. You cannot say it did not occur, nor can you say what you think occurred.

MARCUS: I think since Dr. McClaughry is dealing with negative results in terms of effect of exposure to cold on infectious disease, that we might ask him to comment about the subject of medical-surgical use of hypothermia which has, at least as near as I can make out from a very superficial inspection of surgical literature, also given negative results with regard to infections. Of course, there are circumstances involved here which, again, are mitigating in terms of preventing infection, but I still think it deserves some comment as an aspect of the negative results that you surveyed.

McCLAUGHRY: I think we can have fun with the reports of Dr. Blair and Col. Moncrief. In a pre-planned study, it is possible to measure and record the amount of cold exposure, along with body temperatures, and then follow the incidence and course of infectious diseases. Also, the bacterial and viral flora of persons exposed to cold can be studied.

DIFFICULTIES OF EPIDEMIOLOGICAL STUDIES

MITCHELL: Dr. McClaughry, do you believe that the data are really not available or that they have been in a language that is not translatable to your computer language? You are talking about a reporting situation in which the gaps may be far in excess of the words that you need to translate. A marriage between the reporting of physicians and the computer requires a single common language which permits little or no deviation.

McCLAUGHRY: The data definitely are not available, since they were not recorded. Of course, the question of translation is also a stickler. Much of the present medical data would be classified by the computer people as "soft garbage", and little would be considered hard data. In recordings of blood pressure, for example, Dickinson Richards¹ studied Bellevue Hospital records. No correlation was observed between the values found on the clinical charts, which were random measurements, and those measured under well controlled conditions.

Similarly, in a study done in Julius Comroe's laboratory², an automatic blood pressure recording device gave entirely different blood pressure levels than those measured in the physician's office. There were patients who had been considered non-progressive hypertensive patients who, with the automatic recorders, were found to be normotensive.

BLAIR: I am particularly intrigued with the statement of the negative results of hypothermia, and I stress "hypothermia", not "cold exposure". These are two entirely different matters, of course, but we will have to decide with regard to infections whether or not hypothermia has a specific effect upon the organism involved or on the host himself, if he has any resistance at all.

MARCUS: I questioned some of our thoracic surgeons and found there were four in Salt Lake who engaged in the use of

¹ Richards, D. W., Jr. Personal communication.

² Human, A. L., B. T. Engel, and A. F. Bickford, 1962. Am. Heart, J. 63: 663.

MC CLAUGHRY

hypothermia. They used both the external blanket procedure, and they used extra-corporeal cooling of the blood via the heart-lung machine, and they also told me about the experiences of colleagues, Dr. Lewis from Chicago, and Dr. Duke and Dr. Swan of Colorado. They told me that invariably what these individuals in their own experiences encounter are physiological problems in their patients; problems involving blood clotting dyscrasia, cardiac arrhythmia, and so on.

They lower the temperature of their patients down to as low as 27° C using external blankets for six to thirteen minutes, and use multiple exposures of this type in carrying out their surgical procedures. Invariably, they were surprised when I asked them about infectious disease, and the four I spoke to all said, "Well, of course these patients are all covered by post-operative antibiotic treatment," but in no case were they concerned about postoperative infectious disease. I think you should know about that.

BLAIR: I am very much involved, in addition to my interest in bacteremic problems, in surgery. The fact does remain that we understand and know really very little about what hypothermia does under these circumstances of altering the environment of the bacteria. The University of Minnesota group has been interested; Fisher at Pittsburgh has studied the effects of cold upon so-called host mechanisms. Whatever mechanisms are concerned, we know that bacteria are cleared very readily after injecting and introducing a tremendous number of bacteria into the blood stream. Now, what this really means from the standpoint of the added insult and stress of surgery, anaesthesia and what-not, I don't know. However, purely from the standpoint of the rather empirical criterion of the progress of patients postoperatively, the incidence of infections postoperatively after the use of hypothermia is related only to the surgery. They have been traced without difficulty to errors and faults with reference to techniques in surgery, which all surgeons should know a lot better about, of course. Infections have also been traced to the machinery using extra-corporeal systems of cooling.

DIFFICULTIES OF EPIDEMIOLOGICAL STUDIES

Granted, these are purely a matter of observation. And then with surgeons in our position, one of the most important tools is the ability to look at things from the standpoint of their clinical judgments. At the University of Maryland, Division of Neurosurgery, in the past year almost two hundred patients have been cooled. They are cooling as many patients as we are in cardiovascular surgery, and appear to be our staunchest competitor; the instance of infections is no greater. This is not good scientific evidence, but insofar as taking care of patients is concerned, it does appear that cooling for periods up to about two or three hours to levels of approximately 20°C to 30°C does not increase the incidence of infection following surgery.

MONCRIEF: How about the incidence of infection following profound hypothermia of 10°C ?

BLAIR: The unfortunate fact does remain that the incidence of infection following the use of profound hypothermia which requires a mechanical set-up to cool a patient down this low, does result in a higher incidence of infection than if the patient were simply cooled. Whether this is related to the fact that the patients are cooled to the very profound and potentially lethal levels per se, or whether it is due to the fact that it takes a lot of equipment, we really don't know.

In fact, it does remain that no matter how carefully and how rigidly Lister's ideas are employed today, after an ordinary elective surgical case, if you culture very carefully the surgeon's hands, the instruments, and the nearby drapes, you will always find bacteria in staggering and distressing amounts. But these patients do not develop postoperative infections.

The question remains relatively unanswered, and the only thing I might say about this is that the patients are kept at these profound levels for a very, very brief period of time. In many instances, it does not exceed more than one hour; occasionally two hours. At the University of Maryland -- I guess we were a young, immature group trying to learn -- this is the excuse we give for the fact that we had cooled some patients down to levels of 5°C for periods of about two hours. These were children.

MC CLAUGHRY

They all survived because they were children. Their natural homeostasis was very kind to us.

BERRY: How much adrenal response is there in these patients?

BLAIR: The adrenal response is reduced. Again, this is the difference between induced hypothermia and cold exposure. This is related, of course, to the type of anesthesia used. These people, you know, are all anesthetized. If a barbiturate anesthesia is used, the response of the adrenals is reduced tremendously. Ether anesthesia invokes an increased response for a period of time, but when the patient and the animal are cooled, then the circulating cortico steroid and catecholamines are very much lowered. Hypothermia allays stress response.

BERRY: In other words, hypothermia is almost anti-stress?

BLAIR: That is correct, as opposed to cold exposure.

BERRY: I think these are very important distinctions that we should keep in mind.

MITCHELL: Dr. Blair, where are your prime heat sinks for retaining heat calories in these bodies once you start cooling? You take them down with cooling to 20° C and you maintain this for quite a time. You are now ready to work and you have some tissues there that are rather tremendous heat sinks. They remain warmer, let's say, and then if you move past that to these colder temperatures, do you overcome the ability of that tissue to resist, or do you make it more susceptible, and wherein do you get these infectious processes originally? I am trying to figure out how you approach this.

BLAIR: Are you referring to the instances of cooling either animals or patients down to profound levels in the absence of infections to begin with? Is this what you mean?

MITCHELL: Yes.

BLAIR: First of all, with regard to heat production during

DIFFICULTIES OF EPIDEMIOLOGICAL STUDIES

the induction of cooling, the fact is, of course, that the human being, himself being a heat exchanger and a very inefficient one in many respects, produces a series of problems outside of the infection which have resulted in very serious consequences, sometimes death, and this is because the specific heat of the various tissues are so different. When I say artificial cooling, I am referring to hypoxia in the systems. When a patient is cooled down, he winds up with some very serious gradients in various tissues, the most serious of which is skeletal muscle. The skeletal muscle is not cooled down very much. It is kept quite warm, as a matter of fact. The liver is cooled down quite considerably; also the brain. The net result is that when the cooling process is stopped and the patient is presumably stabilized at a particular level of hypothermia, and this is usually gauged by the esophageal temperature, we begin working on the patient. Of course, a lot of other things are going on, particularly, I think, in the skeletal muscle, which when we start to rewarm the patient, results in a metabolic situation which has made us very, very unhappy; and this is the metabolic acidosis.

Now, I am off the subject that you had raised, Dr. Mitchell. With regard to infections per se, I am not aware of any. They probably have developed and I can only reiterate the attempts of culturing the equipment and, of course, the patient's blood stream, but we have not found any bacteremia. The bacteria, if present, would become overwhelming. This is a matter which would require serious investigation, but then, I personally am not terribly concerned about it because of the time factor. I don't think much is going to happen in one or two hours. These patients are cooled to 10° C in about twenty minutes. They are warmed in about forty-five minutes, but time factors are very short, and I can assure you that the deep level cooling maintained is very brief. Attempts to assay host mechanisms have demonstrated that the period of cooling and rewarming has not produced any longstanding effect on the host mechanisms in handling bacterial infections.

CAMPBELL: The anesthetic must play quite a role here, as in allergic reactions which do not occur during anesthesia.

MC CLAUGHRY

BLAIR: Yes, anesthesia, of course, is a two-edged sword. We are not dealing with hypothermia per se in its native true state by any manner of means. We are dealing with modified hypothermia or, if you will, modified anesthesia, and this is because in reference to anesthesia, it is necessary to anesthetize both humans and experimental subject to a rather deep level. We call this surgical anesthesia, and this is the level primarily at which the reflex mechanisms responsible for maintaining homeothermia are depressed. They have to be depressed. The only instance of which I am aware and in which hypothermia is produced in the absence of anesthesia is in patients who have been treated with hypothermia for bacteremic shock and other problems of that nature, and again I will touch upon that later. These people, of course, are not anesthetized.

MARCUS: Do you treat them with morphine or anything to allay their pain?

BLAIR: Our only experience in this regard, Dr. Marcus, has been with individuals whom we have considered to be refractory to the standard therapy. In the judgment of the physician, these are individuals who have become refractory to very intensive therapy in bacteremic shock; these people are usually comatose. Their reflexes are markedly depressed, and these people are cold. It has been very unusual to observe shivering. As a matter of fact, we use shivering as an index as to when to rewarm the patient. It is a sign that the patient is getting better.

McCLAUGHRY: Dr. Campbell may have indicated one of the very important possibilities here; namely that combining anesthesia with hypothermia may make it possible to sort out some of the factors in pathogenesis of some infectious diseases. In particular, it may provide a means of studying host responses to stress, which are really protective mechanisms, but which may become deranged.

BLAIR: These studies are under way, I might add. Through a contact with the United States Army, the University of Maryland has recently established a clinical shock unit which is devoted to treatment of shock, including bacteremic shock, and one

DIFFICULTIES OF EPIDEMIOLOGICAL STUDIES

of the purposes is to investigate these properties we are talking about and about which we really know so very little, particularly in the human.

McCLAUGHRY: It occurred to me, also, that your description of the situation of the patient with bacteremic shock also bears on this question of stress, because bacteremic shock is one of the things which suppresses those functions. In one sense, you have the anesthesia, anti-shock, and the anti-stress state established by physiological and pathological mechanisms.

BLAIR: That is quite right. It is the pathologic state, the comatose state, essentially. The only thing I am interested in, of course, is controlling the reflex mechanism; that is, shivering. In elective situations, we use anesthesia to depress this, and, of course, anesthesia is a tremendous poison. It poisons a lot of things besides the skeletal muscles.

SCHMIDT: I would like to make reference to some work we did here in Alaska. Our original aim was to determine the incidence of respiratory disease here at Ladd Air Force Base. We had about two thousand subjects. It occurred to us that it might be possible to investigate the influence of cold exposure on the incidence of infection. Accordingly, in taking the histories on these men, we started by asking them if they worked outside or inside. This approach proved to be of little value, because even though their duty stations might have been outside, the men were clothed to the extent that they could not be considered to be cold exposed in the sense of being chilled. We eventually grouped the men according to their squadron activities, thinking that cooks and bakers, for instance, would be less likely to encounter cold exposure than would field maintenance crews. About one third of the 1,985 men studied were in squadrons which we considered would have a higher exposure index. We were unable to demonstrate any significant difference in the incidence of upper respiratory infection attributable to cold exposure. I think we ran up against a stone wall in trying to determine, with any degree of certainty, whether these men were actually cold exposed.

MC CLAUGHRY

BABBOTT: In an AFED report of two or three years ago on winter maneuvers in arctic areas, they reported a great many more cases of heat exhaustion than they did frostbite; so even under field conditions it is very difficult to evaluate cold exposure.

ANDREWES: Williams and Lidwell carried out some experiments on post office workers in Britain, and in comparing those people who were delivering mail out of doors with those who were working indoors, the incidence of respiratory infection was rather greater in those who were working indoors, but of course you can't draw very many conclusions from that because they were so much more exposed to other people, and the outdoor people had very little such contact.

MIRAGLIA: There are several reports in the literature that indicate that people that work indoors and go into cold rooms--butchers, for example--do have a higher incidence of sinusitis and middle ear infections. Taylor and Watrous^{3,4} made studies of this using very small groups of individuals, and there are some examples in Spanish literature which have approximately the same data, but as some of us have already indicated, there are other bits in the literature that seem to contradict this, or at least I found evidence to the contrary.

BLAIR: The matter of respiratory infections, of course, has risen in regard to hypothermia per se in patients and there has been an occasional report of patients who have been cooled who developed pneumonia, but these have been incredibly rare, and I, in the years that I have had the privilege of working in this particular area, have never seen respiratory infection in a patient who has been cooled. As a matter of fact, we have cooled patients who have had pneumonitis of one kind or another and have observed it clearing up during the process of cooling. Of course, these people are receiving antibiotics in treatment, but the hypothermia is not retarding the clearance

³ Taylor, H. M., and L. Y. Dyrenforth. 1938. JAMA 111: 1744-1747.

⁴ Watrous, R. M. 1947. Brit. J. Indust. M. 4: 111-125.

DIFFICULTIES OF EPIDEMIOLOGICAL STUDIES

of the respiratory infection, in any event.

MITCHELL: You are speaking about hypothermia, and the other people have been talking about cold exposure, and it turns out that people exposed to cold up here may be more likely to suffer from heat exhaustion than from cold, and I wonder, maybe, if we should instrument a few people and put them out in the cold.

DRURY: Captain Veghte has considerable data on body temperatures during cold exposure.

MITCHELL: I imagine that there is more stress in thinking about having to go on one of those maneuvers than there is in the experience.

MONCRIEF: In reference to Dr. Marcus's question to Dr. Blair about incidence of infection with induced hypothermia, Dr. Blair mentioned the fact that it is the individuals who have the hypothermia induced by the extra-corporeal circuit that have the increased incidence, if there is any. I think infection is directly proportional to the number of couplings and number of instruments that you have the patient hooked up to; and this, I think, is very well pointed out in our normothermic patients when we put on an extra-corporeal circuit such as extra-corporeal hemodialysis for renal insufficiency.

REINHARD: I am impressed by the fact that the conversation of the last hour has moved us into an area that looks somewhat like a bucket of worms, and I wonder whether or not we are trying to sum up before we have ever heard what each person has to contribute.

ASPECTS OF ARCTIC EPIDEMIOLOGY

Frank L. Babbott, Jr.

University of Pennsylvania
School of Medicine
Philadelphia 4, Pennsylvania

ABSTRACT

Few human pathogens found in arctic populations are localized to this geographic region, although some parasites may have a restricted distribution coinciding with that of their intermediate hosts. The variety of infectious agents is more limited than in temperate or tropical climates. With respect to spread, short chain person-to-person transmission appears more important than dissemination by vector or vehicle. The age distribution of cases and the clinical response of a particular ethnic group to a given disease can usually be explained on the basis of past exposure. Under conditions of normal arctic living, there is little evidence that low environmental temperatures directly affect the ability of the human host to react to antigenic stimuli. Rather, in the Eskimo village, cold is important because of its indirect effect on the way people live.

Epidemiology has been defined as a study of the distribution and determinants of mass disease, or disease as it affects populations. Consequently, our focus is not primarily on the cellular manifestations, nor even on diagnosis and treatment of the individual patient. And yet, we must understand pathogenesis, as well as the clinical picture, if we are to unravel the determinants of the disease as it behaves in populations.

Of course, cold, as a physical agent, can and does directly affect individuals and groups of people. During World War II, cold alone, or cold combined with wet, resulted in the hospitalization of 91,000 U. S. Army personnel in all parts of the world. Seventy-one thousand cases of cold injury occurred in the European Theatre, mostly during the winter of 1944-1945 (Whayne, 1958). At one time in the winter of 1943, frostbite injuries in United States heavy-bomber crews accounted for more casualties than all other causes combined. Gunners in B-17 and B-24 aircraft were especially at risk as they maneuvered machine guns through open "waist ports" while flying at 25,000 to 35,000 feet in temperatures ranging between 25° F and 45° F below

BABBOTT

zero (Davis, 1943). As you realize, the resulting disability was often permanent, with loss of fingers, toes, or even a whole foot. Less severe cases required hospitalization ranging from a few days to many months. So there can be no doubt about the importance of direct injury by short-term exposure to cold, especially in military populations.

But a long-term direct effect of environmental temperature on evolving races of man has also been postulated. Howells points out that ethnic groups arising in the warmest and coldest climates seem to have developed a physique and body mass which permits the maximal dissipation or conservation of heat (Howells, 1960). He contrasts the large surface area of the Sudanese tribesman with the squat, compact build of the pure-blooded Eskimo, and discusses the advantages of each with respect to the physiologic demands of his immediate surroundings. Thus, cold seems to have a direct effect not only on men as individuals, but even on the development of the race itself.

However, the purpose of this symposium is not to discuss cold injury per se, but rather to explore some of the more subtle influences of cold on infectious agents and host responses. In the laboratory, as we will be hearing, it is possible to raise animals, infect them and measure their reactions, all under hypothermic conditions. Likewise, we may propagate the agent at any point on the temperature scale compatible with its survival. Yet Dr. McClaughry has just pointed out the difficulties encountered when we try to undertake similar observations on human populations living in a setting rampant with uncontrolled and even unrecognized variables.

Part of the trouble is that under natural conditions, both men and infectious agents do their best to avoid the less than optimal environment imposed deliberately in the laboratory. We can set up an experimental hypothermic stress and force mice to a compensatory physiologic adaptation, one manifestation of which may be a reduced capacity to resist infection. But when it gets cold at Wainwright or Fort Yukon, the inhabitants merely put on an extra parka or add more fuel to the stove. Only occasionally is a hunter caught on an ice floe or an airman down on the tundra in circumstances where he experiences the same sort of stress required of the laboratory

ARCTIC EPIDEMIOLOGY

mice. Likewise, bacteria and viruses grow best within a rather narrow temperature range, although low ambient temperatures may promote survival. If they are human pathogens, they usually prefer an environment close to 37° C. Fortunately for these organisms, they are seldom forced to adapt to lower temperatures, because their human culture medium does everything possible to maintain thermal homeostasis, regardless of external conditions.

Between 1954 and 1957, I was part of a group under the sponsorship of the Armed Forces Epidemiological Board looking into the transmission of shigella and salmonella infections in various parts of the Arctic. Frequently when hearing of our studies, people would ask how these bacteria withstand such an inhospitable climate. Our answer was that shigella and salmonella grow and multiply at 37° C, and people who inhabit the Arctic maintain the same body temperature as that of their distant relatives in better known parts of the world.

This does not mean that there are not both obvious and unrecognized physiologic responses to cold which may well have a bearing on infectious illness. I am thinking of such things as blood flow and secretory activity of the upper respiratory tract and certain endocrine responses. However, those of us more familiar with field than experimental laboratory studies have hesitated to attribute such unique features as we see to agents evolved under hypothermic conditions or to hosts which have been forced to endure physiologic stress from cold. Rather, if pressed, we are apt to point to such secondary effects of cold as overcrowding within dwellings or permafrost which hinders sanitation. Hopefully, following this conference, our horizons will have been broadened to include more subtle influences which we were previously unable or reluctant to recognize.

AGENTS OF DISEASE IN ARCTIC POPULATIONS

It is convenient, and I believe justifiable, to think of mass disease

BABBOTT

as having three components: the agent, the host population, and the environment. In other words, multiple factors inevitably enter into causation. The task of the epidemiologist is to determine the nature and relative importance of these factors, so that we may know where to apply control measures most effectively.

I would like to talk briefly and in very general terms about some aspects of these three components of mass disease as they relate to the Arctic, and we might begin with a consideration of infectious agents. The first point to be made is that very few human pathogens are strictly localized to arctic areas. Of course, northern populations have been studied less intensively than residents of other regions, and yet I doubt if a totally new bacterium or virus having man as its primary host will be isolated from these people. The same cannot be said with equal confidence for parasites, however, because their life cycles sometimes include intermediate hosts unique to northern latitudes. The second point I'd like to make is that the variety of infectious agents thus far encountered in the Arctic is limited, at least by temperate and tropical standards. This is hardly unexpected, since the same observation applies to arctic flora and fauna generally (Polunin, 1955; Bliss, 1962).

During our intestinal disease studies in Alaska, Greenland, and Lapland, some 4,200 people were examined bacteriologically. Only four types of shigella and five types of salmonella were identified, and all were familiar pathogens. The greatest variety came from a couple of Alaskan village populations which included only 325 people. Among nearly 2,000 well Greenlanders surveyed, the sole pathogenic bacteria found were Sh. sonnei in three carriers and S. paratyphi B harbored by four others (Gordon, 1959). Except for one trematode, Cryptocotyle lingua, the parasites identified in stools of 660 West Greenlanders were well recognized inhabitants of the human gastrointestinal tract. These included E. coli, Endolimax nana, Gardia lamblia, Chilomastix, and, surprisingly enough, Entamoeba histolytica in some 16 per cent (Babbott, 1961).

Hildes and his colleagues (Hildes, 1958, 1959) have carried out serologic surveys in the Canadian Arctic, and Dr. Reinhard will speak shortly about similar work here in Alaska. The agents they identified - poliomyelitis, Coxsackie virus, psittacosis, ECHO virus,

ARCTIC EPIDEMIOLOGY

herpes simplex, influenza and adeno-viruses, to name several - were the same as one might find in many other parts of the world.

But if we are dealing with familiar agents of disease, we are also dealing with agents which may be responsible for widespread sickness and death. The impact of imported illness among Indians and Eskimos is a sad story, and one we hardly have time to document this morning. Suffice it to say that in the 19th century, waves of smallpox, typhoid fever, pneumonia, meningitis and measles swept through susceptible arctic populations, and tuberculosis has been endemic at a high level for decades. In fact, it is only within very recent years that accidents have replaced tuberculosis as the primary killer. Currently, accidents account for approximately a quarter of total deaths in the Eskimos of Alaska and Greenland (Alaska Health Dept., 1962; The State of Health in Greenland, 1959).

As a measure of life threatening forces, you might be interested in a few comparative statistics. In 1959, the mortality rate for Alaskan natives was 9.7 per 1000 population and for Greenlanders, 10.3 per 1000 (Alaska Health Dept., 1962; The State of Health in Greenland, 1959). This is close to the 9.3 per 1000 experienced by the white population of continental United States, or "the lower 48", as Alaskans call it. The birth rate for Alaskan natives that same year was 47.7 per 1000, and for Greenlanders, 51 per 1000; more than twice as high as the birth rate among whites in continental United States (23.1 per 1000). Taken together, the current birth and death rates mean that close to 40 people are being added to each 1000 in the population each year, thus giving Greenland and Alaska one of the highest rates of natural increase in the world. If this annual four per cent increase is maintained, the population will double in less than eighteen years. Considering limited arctic resources, and especially the limited locally available food supplies, it is obvious that the growing population will be more and more dependent upon goods and services brought in from outside.

We will hear much pertinent information about the influence of environment on disease agents in the coming sessions. Under natural conditions in the Arctic, a human pathogen, to survive, must either complete its life cycle within the body of man or some other warm blooded animal, or the agent must be able to withstand exposure to

BABBOTT

cold. Included among sensitive pathogens not having an animal reservoir are the gonococcus, pneumococcus, and many respiratory viruses. *Trichinella* and *echinococcus* typify parasites having an intermediate host, as does rabies, an important disease in the arctic setting.

Dr. Reinhard will have considerable to say about the survival of viruses, but as examples of nonviral agents resistant to environmental stress, we usually think of spore-formers or encysted protozoa. Among the spore-formers, *Clostridium botulinum*, type E, presents an important health problem for Eskimos living along the Bering Sea and northern Labrador. Spores deposited in ocean silt may contaminate marine mammals and subsequently propagate in improperly stored meat, such as seal flippers. Between 1945 and 1960, 44 human cases and 23 deaths were reported (Dolman, 1960). However, studies have shown a rapid die-off of cysts of *Endamoeba histolytica* at temperatures below freezing (Chang, 1954). Although we don't usually regard enteric bacteria as particularly hardy organisms, investigators from the U. S. Army Environmental Health Laboratory conducted some interesting experiments in Fort Churchill, Canada, using fecal samples seeded with *S. typhi*, *S. paratyphi B*, and *Sh. sonnei* (Human Wastes, 1954). These were placed at tundra sites, and recultured periodically from December to July. *Sh. sonnei* could not be recovered after 17 days, but *S. typhi* was grown out after 45 days and *S. paratyphi B* after 135 days. The recovery period for these salmonella organisms was even longer in feces from carriers. They had survived, but not multiplied.

It might be appropriate here to mention modes of transmission under arctic conditions. Summed up briefly, it appears that short chain, person-to-person dissemination is more important than complex, vulnerable spread involving vehicles, vectors, or extra-human reservoirs. Certainly the latter exists, as exemplified by prevalent diseases such as rabies, trichinosis (Thorborg, 1948; Connell, 1948; Bradly, 1950), echinococcosis (Rausch, 1954), diphylobothriasis, and tularemia (Philip, 1962). However, where bacterial intestinal infections would commonly be transmitted through food and water in other parts of the world, these illnesses in the Arctic present an epidemiologic picture much more consistent with contact spread (Gordon, 1959; Fournelle, 1959; Gordon, 1961). The same

ARCTIC EPIDEMIOLOGY

goes for the enteroviruses which Dr. Reinhard will discuss shortly. As a group, respiratory afflictions, both viral and bacterial, are very important, and of course are spread by contact. In terms of mortality for Alaskan natives in 1960, influenza and pneumonia ranked second only to accidents. Tuberculosis and bronchitis were also among the first 10 causes of death (Alaska Health Dept., 1961).

Thus, we may conclude that while the variety of infectious agents is limited, the organisms are, in general, very similar to human pathogens isolated elsewhere. With regard to transmission, short chain person-to-person spread appears at present to be more important than dissemination by vector or vehicle.

Because much detailed attention will be given to agents of disease, I would now like to mention a few factors involving the host.

SOME HOST FACTORS IN DISEASE OF ARCTIC POPULATIONS

Disease in any population may be described in terms of certain host characteristics. One of these is the race or ethnic background of the people under study. At least until very recently, tuberculosis in Eskimos has behaved rather differently than it does in Caucasians. Prevalence was high, and as of 1957, 30 per cent of adults living in the Yukon-Kuskokwim delta region of Alaska had X-ray evidence of past or current pulmonary involvement (Comstock, 1959). The disease ran a much more acute, often fulminating course, with many extrapulmonary lesions (Schaefer, 1959). It is interesting to note that Negroes in continental United States used to exhibit a similar response to tuberculosis (Pinner, 1932), as did Senegalese troops during World War I, living in the same military environment as their French cohorts (Borrel, 1920).

Of course it is often difficult to differentiate between innate host factors of resistance and environment as it influences exposure, particularly when we study human populations. However, Lurie has shown significant family variations in tuberculosis susceptibility

BABBOTT

among rabbits receiving an identical dose of the bacillus (Lurie, 1941). It does not seem unreasonable to postulate a similar diversity of nonspecific resistance for the human animal, even if it is more difficult to demonstrate. In terms of species survival, it is to the advantage of both host and parasite that they live in relative symbiosis. The long-term process of natural selection tends to evolve such a relationship. But regardless of whether one puts emphasis on host or environmental factors in explaining observed differences in disease behavior, it is important to consider and specify all possible attributes of the population under study, including ethnic background.

Age is a second host characteristic, and we often find an unexpected age distribution of cases, especially when infectious illness appears in isolated arctic communities. For example, measles was imported by a Danish sailor to the vicinity of Julianehaab, Greenland, in 1951. (Christensen, 1953). An attack rate of 99 per cent resulted, not just among children, but throughout the general population. Only the very elderly were spared in appreciable numbers, indicating that no measles had occurred in this community in more than sixty years. Likewise, it has been possible to reconstruct the past history of mumps, poliomyelitis and influenza here in Alaska by relating antibody titers to the host characteristic of age (Philip, 1959; Reinhard, 1960; Philip, 1962).

Of course, a very important component of host resistance is the presence of specific antibody, which presupposes prior contact with the antigen. I will speak shortly about the arctic environment as it promotes or inhibits such contact. But let me return for a moment to a point we touched on earlier; that is, under conditions of normal arctic living, we lack epidemiologic evidence to show that low environmental temperatures directly alter the man's ability to respond to an antigenic stimulus. On the contrary, the Alaska Department of Health and Welfare does not differentiate between the Indians of Southeastern Alaska and the Eskimos of Northern Alaska when planning an immunization program, nor do they expect resulting levels of immunity to differ from those seen in continental United States (Pauls, 1962). Likewise, serologic studies following natural infection fail to demonstrate deviation from an expected distribution of antibody titers (Philip, 1959; 1962; Reinhard, 1960).

ARCTIC EPIDEMIOLOGY

The reason for this uniformity in host response under natural conditions appears to be that residents of Barrow or Thule or arctic Lapland so modify their microclimate that they seldom suffer physiologic stress from cold, although they are susceptible to some of its secondary effects.

In the time remaining, I would like to mention briefly certain features of the arctic environment.

ENVIRONMENTAL FACTORS IN DISEASES OF ARCTIC POPULATIONS

The geographer defines the Arctic as that portion of the northern hemisphere lying within the Arctic Circle, which falls at $66^{\circ} 30'$ north latitude. This marks the southern boundary of territory where at least one day each summer the sun never dips below the horizon, and one day each winter it never appears. The characteristic types of terrain are tundra, usually bordering the coast, taiga or inland forested regions, and glacial topography typified by the Greenland ice-cap and parts of the Canadian archipelago.

The climatologist defines the Arctic as that territory lying within the 50°F isotherm, an imaginary line which bounds an area where the average temperature during the warmest month of the year does not exceed 50°F . To the agronomist, concerned with crop potential, the number of frost free days a year is much more important than minimal winter temperatures.

Permafrost is one physical feature of the arctic environment which influences distribution of disease. Obviously when the earth is permanently frozen, subsurface excreta disposal is difficult or impossible, and water must be procured from easily polluted surface sources. A more indirect, but significant affect of permafrost is the restriction it places on agriculture. People inhabiting such an area must rely upon a hunting or fishing economy with its attendant lower standard of living, unless, of course, military establishments or

BABBOTT

industry, such as mining, are available to provide employment.

There is no doubt that cold influences disease behavior in arctic populations, but as I indicated, we have little evidence that under natural conditions, cold of itself directly affects agent pathogenicity or host resistance. Rather, it is important because of the way it modifies the social environment or the way people live. For example, houses must be heated ten or eleven months of the year. Fuel is expensive in terms of either the money or the effort required to procure it. To conserve heat, dwellings are small with consequent overcrowding, and thus conditions are ideal for transmission of respiratory illness.

Studies in other areas have shown that the incidence of enteric disease is influenced more by the availability of water in adequate quantities than by its purity (Hollister, 1955; Schliessman, 1958). Because of the cold, people in arctic regions depend for many months each year on melted ice or snow for their water supply, and this again requires the expenditure of precious fuel. It is not surprising, then, that the level of sanitation is low, and fecal-oral spread of intestinal organisms is easily accomplished. Yet with respect to bacterial enteric illness, low environmental temperatures also serve a useful purpose in that bacterial pathogens are immobilized and killed off in large numbers, and spread by flies is greatly minimized. Cold appears to promote survival, if not multiplication, of viral agents, as Dr. Reinhard will point out.

Another indirect effect of cold on arctic disease has been the limitations it has imposed upon travel until relatively recently. Half a century ago, the inhospitable environment served to protect people from imported pathogens. Now, there are few, if any, communities which experience prolonged isolation. Fortunately, improved communications brings not only more conventional patterns of disease, but also preventative and therapeutic services as well. Therefore, it is highly unlikely that we will see epidemics of the magnitude or severity which characterized the Eskimos' early contact with the outside world. As I have mentioned, death rates among Alaskan natives currently approximate those of their fellow citizens elsewhere. Our problem in the future is not to lower death

ARCTIC EPIDEMIOLOGY

rates, but rather to raise living standards, and this task is not made easier by the rapid population increase.

In closing, I would like to touch upon a few features of the biologic environment. If this meeting had been called in June, we would have been very much aware of the high mosquito density which characterizes an arctic summer. Surprisingly enough, mosquitos have yet to be implicated as disease vectors in this part of the world, though the possibility requires further study, with particular reference to the ARBO viruses. As for animal reservoirs, Hildes and his group at the University of Manitoba, conducting serologic surveys in the Eastern Canadian Arctic, found 15 per cent of 241 Eskimos possessed complement fixing antibodies to psittacosis (Hildes, 1958). Although cross reactions with other viruses, especially trachoma and inclusion conjunctivitis, must be considered, it is very possible these people contacted psittacosis as a result of eating raw birds. I have already referred to trichinosis, echinococcosis and rabies, all of which have mammalian reservoirs. Within recent years, brucellosis has been discovered in both men and reindeer of the Soviet and American Arctic (Cherchenko, 1961; Edwards, 1959; Huntley, 1962).

Finally, to illustrate the importance of understanding arctic ecology, let me mention a Strontium-90 survey conducted here in Alaska with the help of Colonel Fulton (Schulert, 1962). Because of permafrost, radioactive fallout from Soviet nuclear testing accumulates on the surface of the soil where mosses, lichens and other low vegetation grow. These plants provide forage for caribou. A study of caribou antlers in Alaska revealed a concentration of Sr-90 more than ten times that of pooled deer antlers in California. Eskimos in certain areas eat large quantities of caribou, and urine assays in these communities showed that new bone was being laid down with about 12 $\mu\mu\text{c}$ of Sr-90 per gram of calcium, which is four times the average U. S. concentration. This single problem required the interrelating of knowledge concerning meteorology, geology, botany, anthropology, animal ecology, and radiobiology.

BABBOTT

SUMMARY AND CONCLUSIONS

In the short time available, it has been possible to touch upon only a few features which characterize the behavior of human disease in the Arctic. In briefest summary, let me recapitulate:

1) Few human pathogens found in arctic populations are localized to this geographic region, although some parasites may have a restricted distribution coinciding with that of their intermediate hosts.

2) The variety of infectious agents is more limited than in tropical or temperate climates.

3) With respect to spread, short chain person-to-person transmission appears at this time more important than dissemination by vector or vehicle.

4) The age distribution of cases and the clinical response of a particular ethnic group to a given disease can usually be explained on the basis of past exposure.

5) Under conditions of normal arctic living, there is little evidence that low environmental temperatures directly affect either agent virulence or specific host responses. Rather, in arctic populations cold is primarily important because of its indirect effect on the way people live.

I do not believe there is such an entity as "arctic medicine", if by that we mean a unique set of pathologic conditions restricted to this geographic region. However, the ecology of the Far North is distinctive, and may alter the epidemiology of certain diseases. If an illness is to be effectively prevented or controlled, it is important that we understand not only its laboratory and clinical characteristics, but also its behavior in populations.

ARCTIC EPIDEMIOLOGY

LITERATURE CITED

1. Alaska Department of Health and Welfare, 1962. Bureau of Vital Statistics. Personal Communication.
2. Babbot, F. L., Jr., W. W. Frye, and J. E. Gordon. 1961. Intestinal parasites of man in arctic Greenland. *Am. J. Tropical Med. Hyg.* 10: 185-190.
3. Bliss, L. C. 1962. Adaptations of arctic and alpine plants to environmental conditions. *Arctic* 15: 117-144.
4. Borrel, A. 1920. Pneumonie et tuberculose chez les troupes noires. *Ann. Inst. Pasteur* 34: 105-148.
5. Bradly, P. J., and R. Rausch. 1950. A preliminary note on trichinosis investigations in Alaska. *Arctic* 3: 105-107.
6. Chang, S. L. 1954. The survival of cysts of *Endamoeba histolytica* in human feces under low temperature conditions. Annual Report, Commission on Environmental Hygiene, Armed Forces Epidemiological Board 1953-1954.
7. Cherchenko, I. I. 1961. Brucellosis in arctic regions. I. On brucellosis in reindeer. *Zh. Mikrobiol.* 32: 135-159. *J. Microbial, Epidemiol. Immunobiol.* 32: 554-559.
8. Cherchenko, I. I. 1961. Brucellosis in arctic regions. II. On epidemiologic characteristics of a focus of brucellosis in reindeer. *Zh. Mikrobiol.* 32: 118-123.
9. Cherchenko, I. I., and N. I. Samsonova. 1961. Brucella infection in far northern regions. III. Clinical manifestations of "reindeer" brucellosis in man. *Zh. Mikrobiol.* 32: 51-56.

BABBOTT

10. Christensen, P. E., H. Schmidt, H. O. Bang, V. Andersen, B. Jordal, and O. Jensen. 1953. An epidemic of measles in Southern Greenland, 1951. Measles in virgin soil. II. The epidemic proper. *Acta Med. Scand.* 144: 430-449.
11. Comstock, G. W., and M. E. Porter. 1959. Tuberculin sensitivity and tuberculosis among natives of the lower Yukon. *Pub. Health Rep.* 74: 612-634.
12. Connell, F. H. 1948. Trichinosis in the arctic - a review. *Arctic* 2: 98-107.
13. Davis, L., J. E. Scarff, and M. Dickenson. 1943. High altitude frostbite: preliminary report. *Surg., Gynec. and Obst.* 77: 561-575.
14. Dolman, C. E. 1960. Type E Botulism: A hazard of the North. *Arctic* 13: 230-256.
15. Edwards, S. 1959. *Brucella suis* in the Arctic. *Alaska Med.* 1: 41-44.
16. Fournelle, H. J., V. Rader, and C. Allen. 1959. Seasonal study of enteric infections in Alaskan Eskimos. *Pub. Health Rep.* 74: 55-59.
17. Gordon, J. E., and F. L. Babbott, Jr. 1959. Acute intestinal infection in Alaska. *Pub. Health Rep.* 74: 49-54.
18. Gordon, J. E., and F. L. Babbott, Jr. 1959. Acute intestinal infection in the Arctic. *Am. J. Pub. Health* 49: 1441-1453.
19. Gordon, J. E., E. A. Freundt, E. W. Brown, Jr., and F. L. Babbott, Jr. 1961. Endemic and epidemic diarrheal disease in arctic Greenland. *Am. J. Med. Sci.* 242: 374-390.
20. Hildes, J. A., J. C. Wilt, and W. Stackiw. 1959. Neutralizing viral antibodies in Eastern Arctic Eskimos. *Can. J. Pub. Health* 50: 148-151.

ARCTIC EPIDEMIOLOGY

21. Hildes, J. A., J. C. Wilt, and F. J. Stanfield. 1958. Antibodies to adenovirus and psittacosis in Eastern Arctic Eskimos. *Can. J. Pub. Health* 49: 230-231.
22. Hollister, A. C., M. D. Beck, A. M. Gittelsohn, and E. C. Hemphill. 1955. Influence of water availability on shigella prevalence in children of farm labor families. *Am. J. Pub. Health* 45: 354-362.
23. Howells, W. W. 1960. The distribution of man. *Sci. Am.* 203: 112-127.
24. Human Wastes in Arctic and Sub-arctic. Final Report. 1954. Army Environmental Health Laboratory of the Army Medical Service. U. S. Army Chemical Center, Maryland.
25. Huntley, B. E., J. E. Maynard, and R. N. Philip. 1962. Preliminary studies of brucellosis in Alaska. In press.
26. Lurie, M. B. 1941. Heredity, constitution and tuberculosis: an experimental study. Supplement to *Am. Rev. Tuberc.* Vol. 64.
27. Pauls, F. P. 1962. Chief of Laboratories - Southcentral Regional Laboratory, Anchorage, Alaska. Personal Communication.
28. Philip, R. N., B. Huntley, D. B. Lackman, and G. W. Comstock. 1962. Serologic and skin test evidence of tularemia infection among Alaska Eskimos, Indians and Aleuts. *J. Infect. Dis.* 110: 220-230.
29. Philip, R. N., and D. B. Lackman. 1962. Observations on the present distribution of Influenza A/swine antibodies among Alaskan natives relative to the occurrence of influenza in 1918-1919. *Am. J. Hyg.* 75: 322-334.
30. Philip, R. N., K. R. Reinhard, and D. B. Lackman. 1959. Observations on a mumps epidemic in a "virgin" population. *Am. J. Hyg.* 69: 91-111.

BABBOTT

31. Philip, R. N., W. T. Weeks, K. R. Reinhard, D. B. Lackman, and C. French. 1959. Observations on Asian influenza on two Alaskan islands. Pub. Health Rep. 74: 737-745.
32. Pinner, M., and J. A. Kasper. 1932. Pathological peculiarities of tuberculosis in the American Negro. Am. Rev. Tuberc. 26: 463-491.
33. Polunin, N. 1955. Aspects of arctic botany. Am. Sci. 43: 307-322.
34. Reinhard, K. R., and R. K. Gerloff. 1960. Immunity towards poliovirus among Alaskan natives. II. A serologic survey of 47 native communities of western and northern Alaska. Am. J. Hyg. 72: 298-307.
35. Reinhard, K. R., R. K. Gerloff, and R. N. Philip. 1960. Immunity towards poliovirus among Alaskan natives. III. A study of naturally and artificially acquired antibodies against poliovirus among residents of two Bering Sea communities. Am. J. Hyg. 72: 308-320.
36. Rausch, R. 1954. Studies on the helminth fauna of Alaska. XXIV. Echinococcus sibiricensis N. sp., from St. Lawrence Island. J. Parasitol. 40: 659-662.
37. Schaefer, O. 1959. Medical observations and problems in the Canadian Arctic. Canad. M. A. J. 81: 248-253.
38. Schliessman, D. J., F. O. Atchley, M. J. Wilcomb, and S. F. Welch. 1958. Relation of environmental factors to the occurrence of enteric diseases in areas of eastern Kentucky. Public Health Monograph No. 54. (PHS Pub. No. 591).
39. Schulert, A. R. 1962. Strontium-90 in Alaska. Science 136: 146-148.

ARCTIC EPIDEMIOLOGY

40. State of Health in Greenland, The. 1959. Annual Report from the Medical Officer in Greenland. Godthaab, Greenland. (The State of Health in Greenland).
41. Thorborg, N. B., S. Tulinius, and H. Roth. 1948. Trichinosis in Greenland. *Acta Path. et Microbiol. Scand.* 25: 778-794.
42. Whayne, T. F., and M. E. DeBakey. 1958. Cold Injury, Ground Type. Office of the Surgeon General, Department of the Army, Washington, D. C.

DISCUSSION

CAMPBELL: When you study these native populations, in Anaktuvuk Pass and Point Barrow, for example, don't you have to consider the possibility of malnutrition? It seems to me I have heard in the past that vitamins may have an influence on viruses.

BABBOTT: I think that is very important. It is a nonspecific host characteristic which I neglected to bring in, but it is important. The reason I didn't bring it in was that it has been very difficult to do detailed and sound studies on nutrition of these people, particularly in recent years when their diet has been undergoing such rapid change. Dr. Scott, of the Arctic Health Research Center, I know, has worked on certain types of anemias and on vitamin levels. I was not familiar with a very extensive literature on this, and so I did not include it; but it is important.

CAMPBELL: Aren't Eskimos calcium deficient?

BABBOTT: Not that I know of. Do you know anything about that, Dr. Reinhard?

REINHARD: Not in general, but there may be specific people

BABBOTT

whose diet has become so perverted that they might be calcium deficient. This matter of diet is a vexing socio-economic situation here in the northland. For instance, at Anaktuvuk Pass the diet may be highly adequate in years when the caribou are coming through en masse, and in other years, when the caribou hit another way through the mountains, the people's diet may be down to a bare minimum level.

So dietary adequacy fluctuates in these people from time to time. Now, in certain areas where the game animals numbered in the millions in the past have been depleted -- such as the Kuskokwim area -- the people just don't get as many fish, caribou, whales or seals as they did years ago. There the former diet has given way to one supplemented to a large extent by flour and sugar, which isn't especially nutritious. But it is a problem that needs study. It hasn't been studied very well, and I'm glad you brought it up. I might mention one more thing. One of the medical cliches of the past held that Eskimos were hypersusceptible to tuberculosis, but it has been demonstrated by vigorous case findings, hospitalization, and ambulant chemotherapy programs in the past eight years, that the tuberculosis epidemic could be abated rapidly, and I would like to propose that the Eskimos are considerably more resistant to tuberculosis than is generally conceded, or they might have been dead long ago -- wiped out as a race. We have to differentiate between medically underprivileged people and hypersusceptible people. They are not the same.

MITCHELL: Are we talking really and truly about tuberculosis, or are we talking about X-ray evidence of a lung infection which might be or might have been tuberculosis, or a fungus, or something other than, say, the mycobacterium?

REINHARD: We are talking about clinically proven cases of tuberculosis, the statistics of hospital admission, the decrease in positive culture results in various laboratories, the decrease in the malignancy of the cases entering hospitals, and the decrease in death rate. These are all rather positive changes that have occurred very rapidly in the last ten years. In other words, there is proof there. It is not a supposition.

ARCTIC EPIDEMIOLOGY

MITCHELL: The avitaminoses that you are speaking of; would you say that is something that we brought to Alaska? If we hadn't come into Alaska and depleted the food supply, the natives of this area would not be living on flour and sugar.

REINHARD: I think it would be well to get rid of the concept that the white man has consistently done wrong to the Eskimo. After all, the inequities caused by interactions of colliding cultures are part of the normal history of the world, and we have to recognize that they will occur. To go back to the original question, I don't know of any real data on the general occurrence of specific avitaminoses among Alaskan natives.

NUNGESTER: Let's go back just a moment to avitaminosis. Is there any evidence of scurvy in the Eskimo?

REINHARD: I have heard that traditionally, Eskimos were supposed to have no caries, no scurvy, and no body odor.

SCHMIDT: Dr. Babbott, you mentioned that the Russian atomic tests have caused a great deal of Sr^{90} fallout in various parts of Alaska. I was under the impression that there were other countries also testing. Did fallout from these tests not reach Alaska?

BABBOTT: This particular study was done after the first series of Russian tests. I am sure they are not the only ones.

MITCHELL: Dr. Babbott, we could show that people in Florida are taller than the people in Alaska, maybe on the average, and perhaps attribute this to atomic detonation. When you make studies of antlers from animals here in the Arctic, do you find an increased amount of Sr^{90} in those antlers compared with the antlers of animals gathered, say, in California, and sent to Smithsonian prior to the detonation?

BABBOTT: I think a baseline study would be very valuable.

MITCHELL: It certainly would be indicated, because I have been plagued with information of this kind.

BABBOTT

BLAIR: I was most interested in the observations, Dr. Reinhard, of the Eskimos' response to treatment of tuberculosis. There is so much doubt about the change in the organisms with respect to the chemotherapy, the rate of mutation of the different strains and the effect of the various agents they use. Do you have the same problem?

REINHARD: There is a paper in the 1962 AAAS Alaska Science Conference by Shepard of the ADH¹ laboratories which gives a resumé of the decline in positive cultures over the last eight to ten years, and then in surge, in 1962. At the time she postulated that this was due to an occurrence of resisting types. However, there are many other factors that were not dealt with in the paper, or brought under control, such as the possibility that the ADH might have been going through another surge of case findings. Furthermore, the laboratory hasn't been running routine tests for resistance. So far, we don't know whether there is an increase of the resistant types. I don't know whether the Anchorage laboratory² may have better results than this. I wish we could call up Frank Pauls right now.

BERRY: We do have a man here, Dr. Huntley, from the Anchorage Laboratory. Do you have any information on this?

HUNTLEY: No, however, I know that the Alaska Department of Health Laboratory is now doing an antibiotic sensitivity study on positive TB cultures which has resulted in a tremendous increase in the number of cultures requested from field nurses. The study has not been under way long enough (probably 6 to 8 months), therefore, and no data are available on possible resistant strains.

McCLAUGHRY: I'd like to make a comment on Dr. Babbott's remark that there is no such thing as arctic medicine. This has been accepted as a concept by the National Research Council Committee on Tropical Medicines. In attempting to sharpen

¹ Alaska Department of Health.

² Laboratory, Southcentral Region, Alaska Department of Health.

ARCTIC EPIDEMIOLOGY

their definition of what they were concerned with in tropical medicine, they have also come to recognize the phenomenon of medical underprivilege.

ANDREWES: The thing that struck me about Dr. Babbott's paper was the very small amount of evidence there was that cold played any part in the story except indirectly. The effect of cold on the habits and crowding of the people is obviously one consequence, but you get crowding in underprivileged people all over the world, including tropical areas, with the same results. The other thing which so obviously effects the issue is the lack of past experience to particular pathogens causing these outbreaks. Now, particularly in relation to common cold research, we thought it was something of great importance to conduct planned studies on the behavior of isolated communities to see what happened to them when they were isolated and when they made contact with civilization again; and apparently we have missed the bus, because there don't seem to be any isolated communities any more. Even though they have a permanent station on the South Pole, I doubt if we are ever going to be able to get the kind of information we hoped to get.

REINHARD: I don't think the cause is entirely lost. There are some semi-isolated communities right here in Alaska which would provide beautiful study opportunities to a person if he were willing to sit down in the community and test for every virus that came through, but it would be a running fight all the way. You would have to take what the Lord sent you and analyze it without a hope for control on introduction of viruses.

THE ECOLOGY OF ENTEROVIRUSES IN ALASKA

Karl R. Reinhard

Division of Research Grants
National Institutes of Health
Bethesda 14, Maryland

ABSTRACT

The paper considers the following factors; effects of various physical and chemical environments on enteroviruses, the mechanism of enteroviral infections, the carrier state in convalescent and immune individuals, and northern ethnic and social patterns. The background information on the natural history of the enteroviruses is piecemeal. Facts may be drawn from diverse works such as the virology of water and sewage treatment, interferon and the inapparent persistence of viruses in hosts, epidemiological episodes of enterovirus infections, the effects of cold climates on community and household hygiene and sanitation, serological and cultural studies of enterovirus ecology, and morbidity and mortality statistics on northern populations. The author attempts to discern, through this piecemeal evidence, the major determinants of enterovirus ecology in northern areas.

The principal objective of this discussion will be the development of concepts about the natural history of enteroviral infections within the context of the general theme which deals with the influence of cold on host-parasite relationships. To explore adequately the relationship between arctic peoples and their environments and the enteroviruses, we must borrow from such diverse fields as virology, sanitary engineering, meteorology, anthropology, archaeology, epidemiology, and the more general aspects of natural sciences. This brief discussion does not allow an exhaustive treatment of all of these, but if some of the larger issues are clarified, the author's hopes will be fulfilled.

The large Enterovirus group is comprised of particulate agents which are commonly recoverable from the human and animal gastrointestinal tract. These viruses have a ribonucleic acid core; are about 28 millimicrons in diameter; are pathogenic for primates, suckling mice or certain types of mammalian cell tissue culture; and are stabilized by cations against thermal inactivation (Committee on Enteroviruses, 1962). In this group are the polioviruses,

THE ECOLOGY OF ENTEROVIRUSES IN ALASKA

Karl R. Reinhard

Division of Research Grants
National Institutes of Health
Bethesda 14, Maryland

ABSTRACT

The paper considers the following factors; effects of various physical and chemical environments on enteroviruses, the mechanism of enteroviral infections, the carrier state in convalescent and immune individuals, and northern ethnic and social patterns. The background information on the natural history of the enteroviruses is piecemeal. Facts may be drawn from diverse works such as the virology of water and sewage treatment, interferon and the inapparent persistence of viruses in hosts, epidemiological episodes of enterovirus infections, the effects of cold climates on community and household hygiene and sanitation, serological and cultural studies of enterovirus ecology, and morbidity and mortality statistics on northern populations. The author attempts to discern, through this piecemeal evidence, the major determinants of enterovirus ecology in northern areas.

The principal objective of this discussion will be the development of concepts about the natural history of enteroviral infections within the context of the general theme which deals with the influence of cold on host-parasite relationships. To explore adequately the relationship between arctic peoples and their environments and the enteroviruses, we must borrow from such diverse fields as virology, sanitary engineering, meteorology, anthropology, archaeology, epidemiology, and the more general aspects of natural sciences. This brief discussion does not allow an exhaustive treatment of all of these, but if some of the larger issues are clarified, the author's hopes will be fulfilled.

The large Enterovirus group is comprised of particulate agents which are commonly recoverable from the human and animal gastrointestinal tract. These viruses have a ribonucleic acid core; are about 28 millimicrons in diameter; are pathogenic for primates, suckling mice or certain types of mammalian cell tissue culture; and are stabilized by cations against thermal inactivation (Committee on Enteroviruses, 1962). In this group are the polioviruses,

REINHARD

Coxsackie A and B viruses, the ECHO (Enteric Cytopathic Human Origin) viruses, the enteric viruses of animal origin, and the REO (Respiro-enteric) viruses. Why are the enteroviruses important? This is well demonstrated by the kinds of diseases they cause: poliomyelitis, encephalitis, aseptic meningitis, herpangina, pleurodynia, pericarditis, myocarditis, exanthematous fevers, gastroenteritis, and upper respiratory disease. In addition, the enteroviruses cause a variety of systemic diseases which cannot be distinguished as specific syndromes, and which are usually diagnosed clinically as fevers of undetermined etiology (FUE). These agents are responsible for a major proportion of illnesses in children and infants, and are therefore significant in that respect alone. The enteroviruses are ubiquitous, and are frequently recovered from the upper respiratory tract or feces of people who have no overt disease. The following types of human immunological enteroviruses are recognized: poliovirus, three types; Coxsackie A, twenty-three types; Coxsackie B, six types; ECHO, twenty-six types; and REO, three types (Rosen, 1960; Committee on Enteroviruses, 1962). There are, in addition, a number of types of animal origin, particularly bovine and porcine strains. Of the pathogenetic and infectious characteristics, more will be related later.

All of our information on viruses emphasizes that these are obligate parasites; physiologically and metabolically incomplete organisms that must utilize other living animal or plant cells in order to persist and propagate. Undoubtedly, virologists will eventually produce non-cellular media composed of essential enzymes and metabolites for cultivating viruses. In the natural realm, however, viruses grow only in living organisms. Their existence outside of the living host is a passive one. For this reason, considerations of virus ecology are primarily considerations of host ecology plus physical environmental factors favoring passive persistence or dissemination of the virus between the propagation periods in the host. Accordingly, the effects of cold on virus ecology consist of its effects on availability and suitability of hosts for the viruses, and its effects on persistence of viruses in the physical environment outside the host.

From the epidemiological standpoint, there is little reason to believe that direct effects of cold on the human host have significant

ENTEROVIRUSES IN ALASKA

relation to virus ecology. The arctic resident is not a hypothermic individual. Man lives successfully in the Arctic only because he maintains a subtropical micro-climate within his clothing and a temperate climate within his house. The previous speakers have emphasized the fact that the effects of cold environments are indirect; that is, they have a bearing on hygiene, sanitation, social, and individual activity patterns which affect the passage of viruses from host to host. I do not wish to minimize the message of the later discussions of effects of hypothermia on infection. These experiments are important medically from the therapeutic standpoint, and may also lead to basic information on the metabolic and physiological aspects of cellular and systemic resistance to infection; but their relation to the natural history of human disease in arctic areas is difficult to discern. Exceptions to this statement may be furnished by the occasional excessive exposure of people to cold by accident or improvidence, or by the excessive exposure of the upper respiratory tract to very cold air from extreme arctic conditions or overexertion.

The cold climates operate in two, apparently paradoxical manners on the ecology of the human hosts for viruses. First of all, the aboriginal population has been forced to settle in discrete, relatively small, often widely-separated groups, or, in the past, to live a migratory life to exploit the ecology of the basic food animals. Only in certain areas, such as the fish-rich river-valleys of the past, did boreal population groups cluster closely. The bionomics of food resources, therefore, led to isolated human communities, often with discontinuous communication in the colder seasons. This tended to reduce the speed of dissemination of acute infectious disease between communities. When isolation was enforced by armed guards along the trail, as is reputed to have occurred in Northwestern Alaska during the 1918-19 Influenza Pandemic, villages could escape epidemic disease. On the other hand, a cold environment causes close congested living conditions within communities and families. Consequently, a highly infectious epidemic disease spreads rapidly through a village once it is established.

These diverse effects of cold climates on human ecology and communication led to another paradox; i.e., the season in which arctic villagers were more subject to inclement weather was also the time

REINHARD

when they were more free of acute infectious disease. Elder residents of St. Lawrence Island have recounted to the author how, in the "old days", from fall to spring they could expose themselves to chill and fatigue, yet never have a "cold", pneumonia, or other acute infectious disease. However, the first boat of spring arriving from the mainland would bring with it as invisible cargo acute infectious disease; particularly upper respiratory disease. Thereafter, sickness would be common on the island until freezeup, when cessation of traffic from the mainland occurred. Similar experiences were common throughout the Arctic in years past. Many are the accounts of introduction of disease into arctic villages through the advent of people from areas with more concentrated population. Repeatedly, epidemic diseases such as smallpox, measles, influenza, and whooping cough decimated the population in individual villages, affecting young and old alike.

The general epidemiological patterns of the past are not generally applicable to the Arctic today; particularly not in the Western American Arctic. Most villages are in relatively close communication with urban areas because of a well-developed air transport system. There is extensive human traffic throughout the year, and now, therefore, very few villages experience the traditional freedom from acute infectious disease during the colder seasons. Recent epidemiological studies of St. Lawrence Island residents have shown the year-round occurrence of acute infectious disease (Reinhard, 1956). However, this epidemiological shift has not been recognized widely. Perhaps it has been poorly documented. Therefore, in the concepts of the large group of medical and public health professionals in the more populated southerly areas, the Arctic still is the place where people are exposed only sporadically to infectious disease and are hypersusceptible to it when it is introduced. The native arctic population is presumed to be immunologically underdeveloped.

Adherence to these obsolete concepts caused considerable alarm among those concerned with native health when a severe epidemic of poliomyelitis occurred in Anchorage and Fairbanks, Alaska in 1953-54. Previous severe epidemics in Greenland (Fog-Foulsen, 1955) and the eastern Canadian Arctic (Peart, 1949; Adamson, et al., 1949; Johnsen and Wood, 1954) had caused great morbidity and mortality among the native people in those areas. It was feared that similar

ENTEROVIRUSES IN ALASKA

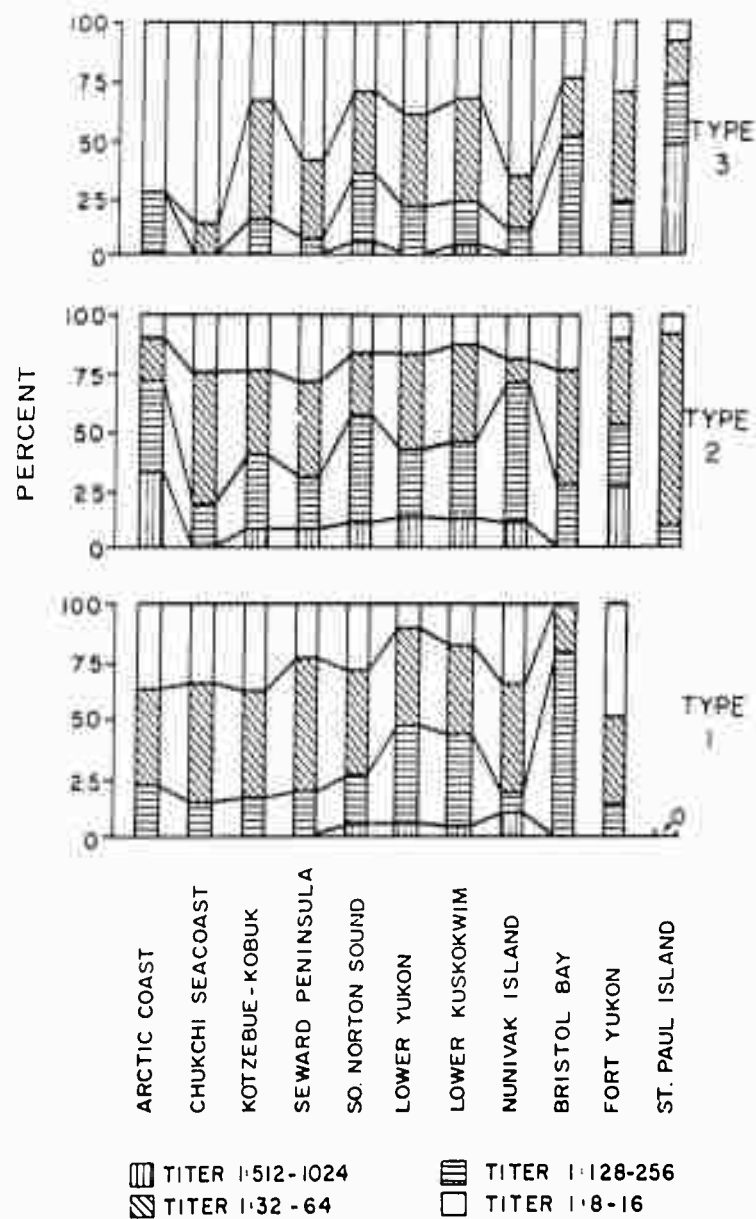


Figure 1. The prevalence of antibodies against the three types of poliovirus in more than 700 native Alaskan males representing 47 villages in 11 geographic localities.

REINHARD

disaster would be caused by extension of the Anchorage epidemic into the native villages of Alaska. However, only one satellite epidemic occurred; the unusual monotypic (Type 3) epidemic on St. Paul Island (Eklund, 1956). Sporadic cases due to various poliovirus types occurred in various native villages, indicating that the polioviruses were active in those areas. But other than the one cited, no recognized epidemics of poliomyelitis occurred in native villages. This intriguing fact led to a series of statistical (Reinhard and Gibson, 1960) and sero-epidemiological (Reinhard and Gerloff, 1960; Reinhard, Gerloff, and Philip, 1960) studies to determine the immunological status of Alaskan natives with reference to poliomyelitis. The data are summarized by a table and two graphs from a publication currently in press.

Table I shows clearly that between 1950 and 1954, Alaskan natives experienced a much lower morbidity rate for poliomyelitis than non-native Alaskans. The disparity was especially great in comparable groups under 15 years of age.

Morbidity Rate per 100,000 per annum

Age Group	Native*	Non-Native
Under 55 years	29	208
5 to 9 years	30	221
10 to 14 years	38	228
15 to 19 years	71	86
20 to 24 years	25	66
25 to 29 years	72	69
30 to 34 years	72	61
35 to 39 years	38	49
40 to 44 years	29	35
45 to 49 years	0	28
50 to 54 years	0	13
55 years and over	0	0

Table I. Comparative poliomyelitis morbidity in Alaskan natives and non-natives, by five-year age groups, based on reports to the Alaska Department of Health, 1950 to 1954. * Adjusted to standard age group proportion.

Figure 1 shows graphically the prevalence of antibodies against the three types of poliovirus in more than 700 native Alaskan males representing 47 villages in 11 geographic localities. The overall prevalences of antibodies against polioviruses were: Type 1, 87 per

ENTEROVIRUSES IN ALASKA

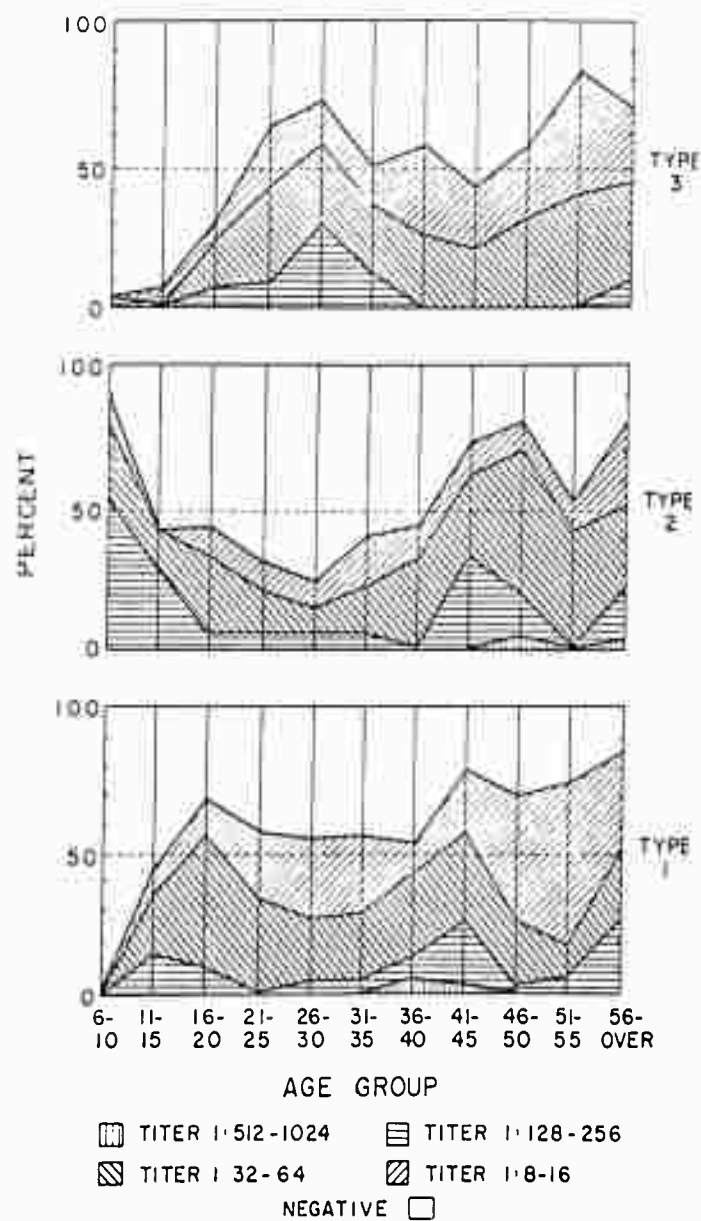


Figure 2. The results of a serological cross-sectional study of the population of St. Lawrence Island.

REINHARD

Village	Date	# of Samples	% of Pop. Represented	# & Type of Isolates in T.C.	# & Type of Isolates in Mice	# Neg. Specimens	# Specs. Yielding 1 Virus	# Specs. Yielding 2 Viruses	+ CPE in TC Virus Not Iso-lated
Napaskiak	July 1956	48	22.9	2-ECHO, Type 7 1-Cox., Type B5	3-Cox. A Type 2	43	6	0	0
Oscarville	Jan. 1957	40	19.1	1-ECHO, Type 5 1-ECHO, Type 6 3-ECHO, Type 10 1-ECHO, Type 11 or 14 1-ECHO, Type 19 6-ECHO, Type undetermined	11-Cox. A Type 3-4	21	14	5	4
	June 1957	80	38.3	6-ECHO, Type 10 8-ECHO, Type 14 2-ECHO, Type 18	1-Cox. A Type 3-4	66	11	3	11
Hooper Bay	Aug. 1958	48	11.2	1 Adenovirus Type 3	0	47	1	0	0
Holikachuk	Nov. 1958	67	68.4	0	0	67	0	0	0
Anvik	Nov. 1958	53	39.8	0	0	53	0	0	0
Shageluk	Nov. 1958	87	61.7	0	0	87	0	0	0

ENTEROVIRUSES IN ALASKA

Iyonek	Oct. 1957	44	28.8	0	0	44	0	0	0
Venetie	Aug. 1958	37	34.3	0	0	37	0	0	0
Arctic Village	Sept. 1958	50	45.0	0	0	50	0	0	0
Beaver	Aug. 1958	22	18.8	0	0	22	0	0	2
Minto	Sept. 1958	24	14.5	1-ECHO, Type 18 2-Cox. A, Type 9	0	21	3	0	0
Ft. Yukon	Sept. 1958	49	8.1	12-Poliiovirus Type 3	0	37	12	0	0
Gambell & Savoonga	Feb. 1958	64	10.1	0	0	64	0	0	0
(St. Lawrence Is-land)	July 1958	33	5.2	0	0	33	0	0	0
	March 1959	58	10.8	15-Poliiovirus Type 3	0	53	15	0	0

Table II. Resumé of virological findings in native village enteric virus surveys. Population statistics for calculation of representation.

REINHARD

cent, Type 2, 92 per cent, and Type 3, 62 per cent. Except for central Alaska (Fort Yukon) and St. Paul Island, the prevalencies of Types 1 and 2 were similar throughout. Antibodies against Type 3, which is generally the rarer of the three known poliovirus types, tended to decrease with increasing geographic latitude and consequent decreasing population density.

Figure 2 summarizes the results of a serological cross-sectional study of the population of St. Lawrence Island. Noticeable in this study were (1) the high prevalence of Type 2 antibodies in the children, (2) the apparent reciprocal relationship between decline in prevalence of Type 2 antibody and increase in Types 1 and 3, and (3) the uniformly high prevalence of antibodies to all three types in the advanced age groups. The results indicated that polioviruses have been endemic, Type 2 more so than Types 1 and 3, and that this situation may have existed for many years.

The epidemiological experiences and serological studies showed, therefore, that Alaskan natives were generally highly immune to polioviruses. Indeed, Alaskan natives appeared to be much more experienced with polioviruses than the urban, non-native Alaskan population. One might speculate, with good reason, that the urban Alaskan epidemics of 1953-54 and earlier years might have been derived from endemic foci in the villages. A relatively higher immunity among natives as compared with non-natives was also found by Adamson and associates in a study of a poliomyelitis epidemic in Whitehorse, Y.T. (1954). Sero-epidemiological studies by Hildes, Wilt and Stackiw (1959) indicate current development of immunity against polioviruses among eastern Canadian natives.

The poliovirus studies stimulated cultural work on the ecology of enteroviruses generally (Reinhard, 1961). Table II presents the results of virological culture of series of stool samples from the residents of native villages. The samplings for these virological surveys were taken opportunistically, as facilitated by field work for the study of other problems. Yet these random surveys yielded a large number of enteroviral isolates. The Napaskiak-Oscarville series are particularly significant because of the large variety of types isolated, the wintertime epidemic of infection, and the long persistence of these agents in so small a population group. The samples from Ft. Yukon in September 1958, and St. Lawrence Island

ENTEROVIRUSES IN ALASKA

Period	Coxsackie Virus																
	Coxsackie Virus, Group A							Group B							ECHO Virus		
	Type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
May-Oct., 1955	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	7
Jan.-Apr., 1956	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	4
June-Nov., 1956	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	10
Jan.-Mar., 1957	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	5
May-Sept., 1957	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	13
Jan., 1958	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	1
July-Nov., 1958	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	6
Jan.-Feb., 1959	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	2
May-Sept., 1959	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	5

Table III. Seasonal occurrence of various types of enteroviruses, Anchorage Pediatric Disease Survey.
 *These Coxsackie A Group viruses were not typed at the time the project was terminated. **These isolates reacted equally with our standard Coxsackie A-3 and A-4 typing serum.

REINHARD

in March 1959, yielded highly significant results in that epidemic of Type 3 poliovirus infection were found to occur in individuals who had received the full course of formalin-inactivated (Salk) vaccine. None of the individuals were ill when sampled.

Table III, derived from a virological study of pediatric disease in Anchorage shows that enteroviruses were found in every season in this urban area in association with overt disease. Because of overburdened laboratory facilities, virological surveys of healthy urban residents were not conducted.

Quite unfortunately, time, facilities, and tenure of research operations did not allow these exploratory studies to mature into more rigorous studies of the natural history of enteroviral infections in the Arctic. It would have been intriguing and profitable to monitor selected representative villages closely by virological and epidemiological means in order to detect the influx, spread, and persistence of various types of enteroviruses in different geographic areas, thereby enabling one to determine the kind of clinical diseases which may have been associated with them. Persistence of virus in various phases of the physical environment could have been studied both naturally and experimentally. However, incomplete as the exploratory studies were, they did demonstrate that enteroviruses, in surprisingly large variety and concentration, share the arctic environment with man...or vice versa!

Confronted with the certainty of the presence and prevalence of enteroviruses in Alaska, one is liable to follow the arctic epidemiological cliché and presume that enteroviruses have been introduced into Alaska perhaps early in this century or even more recently, and have become established and entrenched in the native population before the present days of epidemiological surveillance. However, I would like to present the thesis that enteroviruses might have accompanied man in his migrations into Alaska from the Asian continent milleniums ago. Of course, this thesis is beyond proof, and it is really presented here mostly to combat the arctic epidemiological cliché. There are features of the enteroviruses, however, which suggest strongly that they have been constant companions of the human race since time immemorial.

ENTEROVIRUSES IN ALASKA

Parasitological philosophy has developed the concept that the more successful and probably more ancient host-parasite relationships are those in which the host rarely becomes seriously diseased by the presence of the parasite, and the parasite is able to persist in or on the host despite tissue or humoral reaction. As a group, the enteroviruses qualify eminently as successful parasites. These are the general characteristics of enteroviral infections:

(1) The incidence of infection in an exposed population group is very high.

(2) Infections are frequently asymptomatic, usually benign or transitory, rarely debilitating or fatal to the host.

(3) The host frequently becomes a carrier, disseminating virus for a long period of time, particularly in the feces.

(4) Serologically immune hosts can become reinfected enterically and disseminate virus.

(5) The various types of viruses are antigenically dissimilar to the extent that heterologous immunization of the host is slight or absent, but biological interference between virus types does occur.

In addition, enteroviruses are known to be highly persistent in natural environment, particularly when mixed with organic matter stabilized by cations and in a menstruum of low biological activity such as might be caused by low ambient temperature. We shall speak of each of these points in turn.

There is ample evidence to show that enteroviruses, in general, are spread rapidly. Eklund and Larson (1959), in their study of the January 1954 epidemic of poliomyelitis on St. Paul Island, showed that the infection had pervaded the community to a major extent within 18 days. Their thorough study led them to believe that the virus may have been spread, to a large extent, from oropharyngeal secretions, either by droplets disseminated by coughing and sneezing, or by saliva exchange in the use of common utensils. Bhatt, Brooks, and Fox (1955), in their detailed viro-epidemiological surveillance of poliomyelitis infections in Louisiana, concluded that polioviruses

REINHARD

spread with such facility that the household becomes the epidemiological unit; that is, if one member of a household is known to have poliovirus infection, then all members have probably become infected. Banker and Melnick (1951) first recovered Coxsackie viruses from North Alaskan regions, and Paul et al. (1951) found a high prevalence of antibodies against the isolate in sera from residents of the community yielding the virus. Rosen and associates (1958a; 1958b), in their detailed virological study of enteroviruses in a public child-care hospital, showed that most of the children in the institution became infected within four weeks after the natural introduction of an enterovirus. These are but few of the many descriptions in the literature of explosive epidemics of enterovirus infection. In the Northland, the natural close association of all individuals within a small community facilitates the rapid dissemination of highly infectious agents.

Although enteroviruses are highly infectious, the likelihood that infection will result in severe disease is relatively small. In the St. Paul Epidemic cited previously, 322 Aleuts were involved and the epidemiological evidence indicated no previous experience with Type 3 poliovirus. Of these, less than 2 per cent had severe pathological involvement, and 0.9 per cent died from central paralysis. Eleven (3.4 per cent) had symptoms of benign (aseptic) meningitis, and 4.6 per cent had minor, transitory indisposition. The enterovirus ecological surveys of native villages previously cited, in which large proportions of the individuals sampled yielded virus, had no relation to epidemiological episodes of recognizable disease. The studies of child welfare institutions (Rosen et al., 1958a; 1958b) demonstrated high incidence of enterovirus infection yielded no clear picture of association of these viruses with specific disease. Some studies have shown enteroviruses other than polioviruses to be the cause of epidemics of distressing diseases such as pleurodynia and herpangina (Huebner et al., 1951; Huebner et al., 1953). The general situation, however, is that a large proportion of people who become infected with enteroviruses do not have overt symptoms of disease. This led in the early years of enterovirus discovery to the term "viruses in search of disease" or "orphan" viruses (Symposium, 1957). These clichés have now passed out of common usage, but the fact remains that enteroviruses are capable of utilizing human populations as habitation without production of a high incidence of

ENTEROVIRUSES IN ALASKA

Duration of Excretion of Virus

Virus Type	Type Specimen	Usual Range	Extreme	Author & Ref.
Coxsackie A-2	Feces	9 to 47 days	76 days	Huebner, et al.
Coxsackie A-21	Oropharynx	16 to 40 days	40 days	Johnson, et al.
ECHO 7	Feces	1/2 to 1 1/2 months	3 1/2 months	Heinigst, et al.
ECHO 20	Feces	1 to 4 weeks	6 weeks	Rosen, et al.
Various Types	Feces	1 to 3 weeks	---	Rosen, et al.
Poliovirus	Feces	51 days average	---	Gelfand, et al.

Table IV. Reports of duration of virus excretion after infection by various enteroviruses.

ENTEROVIRUSES IN ALASKA

Duration of Excretion of Virus

Virus Type	Type Specimen	Usual Range	Extreme	Author & Ref.
Coxsackie A-2	Feces	9 to 47 days	76 days	Huebner, et al.
Coxsackie A-21	Oropharynx	16 to 40 days	40 days	Johnson, et al.
ECHO 7	Feces	1/2 to 1 1/2 months	3 1/2 months	Heinigst, et al.
ECHO 20	Feces	1 to 4 weeks	6 weeks	Rosen, et al.
Various Types	Feces	1 to 3 weeks	---	Rosen, et al.
Poliovirus	Feces	51 days average	---	Gelfand, et al.

REINHARD

of serious disease. Even the poliovirus, the most dreaded of the enteroviruses, causes extensive pathology or death in only a very small proportion of their hosts. This fact aids in the persistence of enteroviruses; but it also makes their true bionomic status a difficult study because of the cryptic nature of their activity.

It is characteristic of enterovirus infection that the virus may be present in large quantities in oropharyngeal secretions as well as feces and intestinal secretions. Lymphoid tissue and mucosal epithelium may be the site of propagation of virus. Usually, oropharyngeal samples become negative for virus soon after the acute stage of the disease. A notable exception to this is the case of Coxsackie A-21 infection, in which virus has been found in pharyngeal swab specimens as long as 40 days after onset (Johnson et al., 1962). In most enterovirus infections the virus may be shed in feces for several weeks. Table IV shows duration of enteroviral excretion as demonstrated by a number of workers. Rather consistently, enteroviruses have been demonstrated in the feces of a significant proportion of individuals a month to six weeks after onset of enteroviral infection (Rosen et al., 1958a; 1958b; Johnson et al., 1962; Huebner et al., 1950). This means that the viruses may persist for a month in people whose serum antibodies have reached immunologically effective levels. The true duration of gastrointestinal infection is not known, for recent studies have shown that treatment of feces with freon, which dissociates antigen-antibody complex, may extend greatly the period in which virus can be detected in the healthy or convalescent carrier (Howe, 1962). This prolonged carrier period makes the isolation of villages in cold climates less effective in the prevention of the introduction of enteroviruses, and also helps to maintain the viruses within village and local area populations.

Since the advent of the vaccines, both the formalin-inactivated and attenuated types against poliomyelitis, it has become quite apparent that circulating antibodies against polioviruses are no sure indication of permanent, solid immunity. Serologically-immune individuals can become reinfected with polio viruses homologous to the serum antibodies and can excrete virus (Horstmann et al., 1957; Fox et al., 1958; Gelfand et al., 1960). These reinfections are usually limited to the enteric tract and apparently are pathologically benign. However, virulent virus may be excreted for extended periods,

ENTEROVIRUSES IN ALASKA

leading to infection of susceptible individuals in the environment. Our own data, previously cited, on the recovery of Type 3 poliovirus from previously-immunized children in Ft. Yukon and St. Lawrence Island, comprise a modest corroboration of this infectious potential of enteroviruses. Recent work indicates that other enteroviruses may have this same infectious potential (Henigst et al., 1961). In view of the prolonged carrier state and potential for enteric infection of serologically-immune individuals, it is not surprising that enteroviruses can persist in small semi-isolated groups of arctic residents. The data graphed on Figure 2 can be interpreted to indicate that endemic poliovirus infection had persisted among the St. Lawrence Islanders for many years, despite the group's relatively small current size of 600 individuals and less in previous decades.

Although enterovirus groups may have some basic antigenic similarity (Halonen et al., 1959; Melnick, 1955; Wenner et al., 1956), they are more dissimilar in antigenic composition in that an antibody stimulated by infection with one kind of enterovirus may not protect against infection by other types (Rosen et al., 1958a; 1958b). The earliest analyses of enteroviral antigenicity were made of polioviruses. Here it was found that Type 2 poliovirus shares antigens with both Types 1 and 3, and Type 2 antibodies may protect against infection by Types 1 and 3. But Types 1 and 3 have little antigenic similarity and antibodies against them rarely cross-protect (Hammon and Ludwig, 1957; Wilt et al., 1958; Faro, 1959). This situation is even more diversified among the large Cocksackie and ECHO virus groups. Consequently, serial infections by different enteroviruses occur. However, simultaneous, dual, or multiple infection is rare. This is due to the mechanism of non-specific biological interference mediated, according to recent discovery, by the host-produced Interferon (Wagner, 1960; Baron and Isaacs, 1961). Thus, one virus infection may produce a transitory non-specific reaction of the host which renders the latter insusceptible for a period of time to other viral infections. These phenomena, the diversity of antigenicity and biological interference, in combination, tend to extend the period of time in which an introduced heterologous group of enteroviruses remain active in a given population group, since they limit superinfection but allow serial infection by different types.

Type of Water	Storage Temp.	Time
1. Distilled water "	8° C 20° C	More than 272 days 41 to 135 days
2. Water from unpolluted river	20° C	Less than 4 days
3. Water from moderately polluted river	8° C 20° C	12 to 16 days 6 days
4. Same as 3 but autoclaved before addition of virus	8° C 20° C	151 to 171 days 102 days
5. Sewage	8° C 20° C	50 days 20 days

Table V. Persistence of Coxsackie viruses in various types of waters. Time required for thousand-fold reduction of infective virus titer.

ENTEROVIRUSES IN ALASKA

Considering the foregoing facts, it is not surprising that the few exploratory studies conducted thus far have shown that enteroviruses are and have been endemic in arctic areas. However, the situation is not uniform throughout the Arctic; for example, severe poliomyelitis epidemics have occurred among natives in the eastern Canadian Arctic, in Greenland, and on St. Paul Island, yet enterovirus infections have occurred endemically and almost cryptically in most areas of the western American Arctic. The comparative isolation of communities in these diverse areas, due to differing transport and economic factors, may have much to do with the disparity of their epidemiological experience. It would be highly desirable to determine how long specific enteroviruses could remain endemic in single isolated villages. At present, our experimental approaches and methods may not be equal to the task. We do not understand sufficiently the role of Interferon in cryptic infection. We have inadequate information on the pathogenetic mechanism of the carrier state in convalescents and reinfection of serologically-immune hosts. Virological cultural methods are not adequate to recover cryptic or sparse viral flora with qualitative or quantitative reliability.

We will turn from the indirect effects of cold climates on enteroviral ecology which are mediated by bionomics of the host, and consider the direct effects on the persistence of viruses in the physical environment. Salient in this respect are the studies of a number of workers who have been concerned with the presence of enteroviruses in sewage and in contaminated water supplies. Clarke and associates (1956, 1959; Taft Report) have shown that Cocksackie viruses survive long in pure waters with low biotic content and activity. In temperate waters with high biotic activity and little or no pollution, the survival of Cocksackie viruses was short. With increasing organic pollution and consequent decrease in aerobic biotic activity, the longevity of Cocksackie virus increased greatly. Chang (cited by Clarke) found that Cocksackie virus stored in 10 per cent sewage in water at 10° C survived for 440 days. Table V presents some of the data from several publications dealing with viability of enteroviruses in water and sewage, and which serve to illustrate the foregoing statements. Experimentally, low ambient temperatures were found to extend the survival of enteroviruses in natural waters and sewage. The data in Table VI, extracted from

REINHARD

	Unpolluted River Water			Moderately Polluted River Water			Sewage		
	28° C	20° C	4° C	28° C	20° C	4° C	28° C	20° C	4° C
Poliovirus 1	17	20	27	11	13	19	17	23	110
ECHO 7	12	16	26	5	7	15	28	41	130
ECHO 12	5	12	33	3	5	19	20	32	60
Cox. A 9	8	8	10	5	8	20	6	--	12

Table VI. Effect of temperature on survival of various enteroviruses in various kinds of natural waters. Average time in days for 99.9 per cent reduction in viable virus. (Ref. 39) Clarke, Berg, Kabler and Chang.

ENTEROVIRUSES IN ALASKA

publications of the Robert A. Taft Sanitary Engineering Center, illustrate this conserving effect of low temperatures on enterovirus viability. They show that viability is enhanced further by gross organic pollution. Type 1 poliovirus and ECHO Type 7 persist two to three times longer at 4° C than at 28° C in relatively unpolluted water, but in sewage poliovirus persisted seven times and ECHO-7 four and a half times longer at the lower temperature. The preserving effect of cold storage was not nearly so marked with Coxsackie A-9 and ECHO-12 as with the first two agents.

A few laboratory studies have given some information on persistence of viability of enterovirus cultures at various temperatures. In one investigation of viability of viruses in tissue culture held at 37° C, the following half-lives were observed: Poliovirus-1, 47 hours; ECHO-1, 24 hours; ECHO-4, 18 hours; ECHO-6, 40 hours; ECHO-9, 19 hours; and ECHO-20, 2.5 hours (Lehmann-Grube and Syverton, 1959). In another study, ECHO-20, which was quite short-lived at 37° C, remained fully viable for a year when stored at -20° C (Rosen et al., 1958a). Data kindly provided by Dr. H.G. Cramblett (1962) showed that certain enteroviruses would survive six to eleven times longer at 20° C, and twenty to sixty times longer at 5° C than they did at 37° C. The presence of cells and organic debris enhance viability. Although freezing is a common means of long-term preservation of viruses in laboratory procedures, little is known of a definitive nature of the effects of successive freezing and thawing. Generally, it is considered deleterious to virus viability but definitive information on this point gained in controlled experiments would be desirable.

In general, the experimental evidence shows that low ambient temperatures, high organic content, and low biological activity favor the long-term persistence of enteroviruses in aquatic medium. These favorable conditions could be provided by the haphazard human waste disposal methods of many small arctic communities.

Limited experiments have shown that soil absorbed large quantities of poliovirus; it remained viable for three weeks in natural soil and for six weeks in previously sterilized soil (Murphy et al., 1958). These experiments were carried out at 30° C, but one could expect much longer survival of enteroviruses in soils at lower environmental temperatures. The information available is sketchy,

REINHARD

and much more extensive experiments should be conducted in which the wide variance in soil character and chemical composition are considered.

On the basis of preceding information, we can reasonably expect that the physical environment in arctic communities should be capable of maintaining viral contamination for extended periods of time. As a further complication, the low ambient temperatures of most of the seasons would tend to hinder means of eliminating pollution with viruses, for it is known that the virucidal activity of halogens is much reduced at low temperatures (Clarke and Kabler, 1954; Clarke et al., 1956). Clearly, enterovirus activity in arctic communities presents challenging issues to the microbiologist, epidemiologist, and sanitary engineer. Imaginative and thorough research would be required to clarify these issues.

The scope and depth of virological and immunological research in recent years have forced a revision of many a time-honored epidemiological or biologic concept. The revisions have occasionally been drastic. In accordance with progress, it would be well to re-examine critically the current concepts of arctic epidemiology and change them to conform with the facts of natural history as they are discerned by more subtle and penetrating scientific approach and methodology. We are, however, confronted with two conditions that are conducive to investigational inadequacy. First is the relatively undeveloped condition of arctic biomedical research in that it has been accustomed to dull, often obsolescent tools, and has been carried out in piecemeal, often superficial fashion. Second is the condition of the arctic human community; it is in a state of rapid change socially, culturally, demographically, and economically. The scientific approaches must be equal to the task of discerning the forces which produce the changes as well as recording the changes quantitatively and qualitatively.

The Arctic offers fabulous opportunities for imaginative, technologically-solid epidemiological research. The unique facilities of the Arctic are the small villages with well defined, fairly stable populations. These villages can serve as convenient, easily comprehended population study groups. The semi-isolation offers excellent opportunity for the controlled study of natural introduction,

ENTEROVIRUSES IN ALASKA

pathogenesis, persistence, and disappearance of infectious agents. The people of these villages are pleasant, cooperative and highly reliable when their individuality and dignity are respected. They prize the opportunity to participate intelligently.

In like manner, the individuality of the microbial agent must be respected. The natural history of enteroviruses is not equivalent to that of Br. abortus. Each virus group, each bacterial species, as well as each environment must be approached in a manner free of preconception in order to derive the utmost in objective information. There is no doubt that dedicated, persistent application of progressive approaches and methodology to the problems of arctic diseases will uncover a large fund of information which would not only contribute in application to the health of arctic residents, but which would also yield a greater fundamental understanding of the natural history of diseases.

LITERATURE CITED

1. Adamson, J. D., Malcolm R. Bow, and E. H. Lossing. 1954. Poliomyelitis in the Yukon. *Canad. J. Pub. Health* 45: 337-344.
2. Adamson, J. D., J. P. Moody, A. F. W. Peart, R. A. Smillie, J. C. Wilt, and J. W. Wood. 1949. Poliomyelitis in the Arctic. *Canad. Med. Assoc. J.* 61: 339-348.
3. Banker, D. D., and J. L. Melnick. 1951. Isolation of Coxsackie virus (C virus) from North Alaskan Eskimos. *Am. J. Hyg.* 53: 383-390.
4. Baron, S., and A. Isaacs. Interferon and natural recovery from virus diseases. *New Scientist* 11: 81-82.

REINHARD

5. Bhatt, P. N., M. Brooks, and J. P. Fox. 1955. Extent of infection with poliomyelitis virus in household associates of clinical cases as determined serologically and by virus isolation using tissue culture methods. *Am. J. Hyg.* 61: 287-301.
6. Clarke, N. A., G. Berg, P. W. Kabler, and S. L. Chang. Human enteric viruses in water: source, survival and removability. Mimeo report-Robt. A. Taft. San. Eng. Ctr., USPHS, Cinn., Ohio.
7. Clarke, N. A., and S. L. Chang. 1959. Enteric viruses in water. *J. Am. Water Works Assn.* 51.
8. Clarke, N. A., and P. W. Kabler. 1954. The inactivation of purified Cocksackie virus in water by chlorine. *Am. J. Hyg.* 59: 119-127.
9. Clarke, N. A., R. E. Stevenson, and P. W. Kabler. 1956. Survival of Cocksackie virus in water and sewage. *J. Am. Water Works Assn.* 48.
10. Clarke, N. A., R. E. Stevenson, and P. W. Kabler. 1956. The inactivation of purified Type 3 adenovirus in water by chlorine. *Am. J. Hyg.* 64: 314-319.
11. Committee on Enteroviruses, National Cancer Institute, NIH, 1962. Classification of human enteroviruses. *Virology* 16: 501-504.
12. Eklund, Carl M., and Carl L. Larson. 1956. Outbreak of Type 3 poliomyelitis on St. Paul Island, Alaska. *Am. J. Hyg.* 63: 115-126.
13. Faro, S. N. 1959. Recurrent paralytic poliomyelitis. *New England J. Med.* 260: 1177-1179.
14. Fog-Poulsen, M. 1955. Poliomyelitis in Greenland. *Danish Med. Bull. (Kbh.)* 2: 241-246.

ENTEROVIRUSES IN ALASKA

15. Fox, J. P., H. M. Gelfand, D. R. LeBlanc, and D. F. Rowan. 1958. The influence of natural and artificially induced immunity on alimentary infections with poliovirus. *Am. J. Pub. Health* 48: 1181-1192.
16. Gelfand, H. M., D. R. LeBlanc, J. P. Fox, and D. P. Conwell. 1957. Studies on development of natural immunity to poliomyelitis in Louisiana, part II. *Am. J. Hyg.* 65: 367-385.
17. Gelfand, H. M., D. R. LeBlanc, Louis Potash, D. I. Clemmer, and J. P. Fox. 1960. The spread of living attenuated strains of polioviruses in two communities in southern Louisiana. *J. Pub. Health* 50: 767-778.
18. Halonen, P., L. Rosen, and R. J. Huebner. 1959. Homologous and heterologous complement fixing antibody in persons infected with ECHO, Coxsackie and poliomyelitis viruses. *Proc. Soc. Exp. Biol. Med.* 101: 236-241.
19. Hammon, W. McD., and E. H. Ludwig. 1957. Possible protective effect of previous Type 2 infection against paralytic poliomyelitis due to Type 1 virus. *Am. J. Hyg.* 66: 274-280.
20. Henigst, W. W., H. M. Gelfand, D. LeBlanc, and J. P. Fox. 1961. ECHO virus Type 7 infections in a continuously observed population group in southern Louisiana. *Am. J. Trop. Med.* 10: 759-766.
21. Hildes, J. A., J. C. Wilt, and W. Stackiw. 1959. Neutralizing viral antibodies in Eastern Arctic Eskimos. *Canad. J. Pub. Health* 50: 148-151.
22. Horstmann, Dorothy M., J. R. Paul, J. L. Melnick, and J. V. Deutsch. 1957. Infection induced by oral administration of attenuated poliovirus to persons possessing homotypic antibody. *J. Exp. Med.* 106: 159-177.
23. Howe, Howard A. 1962. Detection of poliovirus in feces of chimpanzees during late convalescence by means of freeze-thaw. *Proc. Soc. Exp. Biol. Med.* 110: 110-113.

REINHARD

24. Huebner, R. J., C. Armstrong, E. A. Beeman, and R. M. Cole. 1950. Studies of Coxsackie viruses. JAMA 144: 609-612.
25. Huebner, R. J., R. M. Cole, E. A. Beeman, J. A. Bell, and J. H. Peers. 1951. Herpangina-etiological studies of a specific infectious disease. JAMA 145: 628-633.
26. Huebner, R. J., J. A. Risser, J. A. Bell, E. A. Beeman, P. M. Beigelman, and J. C. Strong. 1953. Epidemic pleurodynia in Texas. New England J. Med. 248: 267-274.
27. Johnsen, H. V., and J. W. Wood. 1954. An outbreak of poliomyelitis at Maguse River, N.W.T. Canad. J. Pub. Health 45: 16-17.
28. Johnson, Karl M., Henry H. Bloom, Maurice A. Mufson, and Robert M. Chanock. 1962. Acute respiratory disease associated with Coxsackie A-21 virus infection. I. Incidence in military personnel: observations in a recruit population. JAMA 179: 112-119.
29. Lehman-Grube, F., and J. T. Syverton. 1959. Thermal stability of ECHO viruses in cell culture medium. Am. J. Hyg. 69: 161-165.
30. Melnick, J. L. 1955. Antigenic crossing within poliovirus types. Proc. Soc. Exp. Biol. Med. 89: 131-133.
31. Murphy, Wm. H., Jr., O. R. Eylar, E. L. Schmidt, and J. T. Syverton. 1958. Absorption and translocation of mammalian viruses by plants. Virology 6: 612-622.
32. Paul, J. R., J. T. Riordan, and L. M. Kraft. 1951. Serological epidemiology: antibody patterns in North Alaskan Eskimos. J. Immunol. 66: 695-713.
33. Peart, A. F. W. 1949. An outbreak of poliomyelitis in Canadian Eskimos in wintertime. Canad. J. Pub. Health 40: 405-417.

ENTEROVIRUSES IN ALASKA

34. Reinhard, Karl R. 1956. Demographic and preliminary epidemiological studies of the people of St. Lawrence Island, Alaska. Proc. Med. and Public Health Sect., 7th Alaska Science Conference, Juneau. (Not printed in toto. Copies may be requested.)
35. Reinhard, Karl R. Notes on the ecology of enteroviruses in western arctic and subarctic regions of North America. XIII Northern Conf. on Pathol. Microbiol., Turku, Finland, and 12th Alaska Sci. Conf., 1961. To be published in JAMA.
36. Reinhard, Karl R., and R.K. Gerloff. 1960. Immunity toward poliovirus among Alaska natives. II. A serologic survey of 47 native communities of western and northern Alaska. Am. J. Hyg. 72: 298-307.
37. Reinhard, Karl R., R. K. Gerloff, and Robert N. Philip. 1960. Immunity toward poliovirus among Alaska natives. III. A study of naturally and artificially acquired antibodies against poliovirus among residents of two Bering Sea communities. Am. J. Hyg. 72: 308-320.
38. Reinhard, Karl R., and Harry V. Gibson. 1960. Immunity toward poliovirus among Alaska natives. I. Comparative reported incidence of clinical poliomyelitis in Alaska natives and non-native residents. Am. J. Hyg. 72: 289-297.
39. Rosen, Leon. 1960. Serologic grouping of reoviruses by hemagglutination-inhibition. Am. J. Hyg. 71: 242-249.
40. Rosen, Leon, James H. Johnson, Robert J. Huebner, and Joseph A. Bell. 1958a. Observations on a newly recognized ECHO virus and a description of an outbreak in a nursery. Am. J. Hyg. 67: 300-310.
41. Rosen, Leon, Joseph A. Bell, and Robert J. Huebner. 1958b. A longitudinal study of enteroviral infections in young children. Proc. of 6th Intl. Cong. on Tropical Med. and Malaria. V: 5-13.
42. Symposium. 1957. Viruses in search of disease. Ann. N.Y. Acad. Sci. 67: 209-446.

REINHARD

43. Wagner, R. R., and A. H. Levy. 1960. Biochemical aspects of microbial pathogenicity. *Ann. N. Y. Acad. Sci.* 88: 1021-1318.
44. Wenner, H. A., P. Kamitsuka, and M. Lenahan. 1956. A comparative study of Type II poliomyelitis viruses. II. Antigenic differences relating to 18 Type II strains. *J. Immunol.* 77: 220-231.
45. Wilt, J. C., W. Stackiw, J. A. Hildes, E. Taylor, and A. J. W. Alcock. 1958. The immune status of poliomyelitis patients. *Canad. Med. Assoc. J.* 78: 32-34.

DISCUSSION

MARCUS: I have great fear of the Jovian wrath that falls on people who ask questions in the field of enterovirus and polio, and I have to confess, in asking this question, I am very ignorant on the whole subject. I am a little confused on a point here, though. You insinuated, I think, Dr. Reinhard, that a person who has had an enteroviral disease can recover from it and still excrete this virus as a carrier?

REINHARD: For a period of several months. We don't know actually how long.

MARCUS: This is an active infection, but it is somewhat similar in nature to what is done in immunizing with oral virus against an enterovirus. If nobody is looking, I will say it, polio-virus. I read in Readers Digest and Time that when you immunize by this means, you destroy the excretion of virus, so you don't pollute the water supply which comes from the sewage that you don't get back from the tap.

REINHARD: I wonder how well some of these concepts will stand the test of time?

MARCUS: I see. In other words, you feel that perhaps some of

ENTEROVIRUSES IN ALASKA

the statements that are made are not based on thoroughly tested data?

SULKIN: Not exactly. Actually, while there might be a persistence of the attenuated strain, the concept is that the very presence of the attenuated strain would prevent wild type virus from invading the host. So, you are dealing with two different agents; one interfering with the invasion of the host by the other.

REINHARD: You are speaking about biological interference?

SULKIN: Yes, this is precisely the mechanism which, it is hoped, will operate as a result of oral immunization.

MARCUS: And when the excretion of the avirulent strain ceases, you would say, then, the individual is susceptible once again?

WALKER: He is considerably more resistant to infection, even in the gut.

MARCUS: Is this true of the other enteroviruses?

WALKER: They can be reinfected with other antigenic strains, but they must be more resistant.

MITCHELL: If you had virus particles of the so-called attenuating type and the so-called wild type, equal in number, and you placed them before the susceptible cells, which would the cell choose? Would it choose the attenuated, or is the attenuated virus more aggressive than the wild type?

SULKIN: The point is, by introducing attenuated virus, that attenuated virus will replicate in the most desirable part of the body for that organism, which happens to be the alimentary canal, so that you no longer have equal numbers.

MITCHELL: It is already there and usually has the advantage of an increased population. A good big man can always whip a small man.

REINHARD

SULKIN: There are two factors to consider. One is the replication of attenuated virus, and the other is that it is conferring local immunity so that small doses of a wild type virus cannot gain a foothold in the host.

REINHARD: I'd like to propose an additional thought. You remember that in the St. Lawrence study the graphs of Types 1, 2, and 3 poliovirus antibodies indicated interference between the three types in the chronology of initial infection and immunogenesis. (See Figure 2 in manuscript.) Secondly, there was a fifteen to twenty year periodicity between resurgence in prevalence of antibodies, correlated with increase in titers. I wish we had more extensive data to test the validity of these phenomena. I am wondering, however, whether these graphs do not give a true picture of the natural decline of antibodies in an endemically exposed population to the point where reinfection and reinforcement of antibodies takes place.

METCALF: Keeping in mind the so-called doctrine of original antigenic sin¹ and the experiences gained with influenza viruses, is there anything in this data which could or might be interpreted as heterological boosting of one type by another? Does that happen in the polio group?

REINHARD: Not in type-specific antibodies. Does anybody else have any information?

SULKIN: Heterotypic antibody responses have been observed in vaccinated individuals as well as following natural infection with poliovirus. Some time ago Sabin² described the transitory appearance of Type II neutralizing antibody in patients infected with Type I poliovirus and suggested that these two types shared a common antigen.

REINHARD: This would be due to actually shared antigens.

¹ Davenport, F. M., A. V. Hennessy, and T. Francis, Jr. 1953. J. Exp. Med. 98: 641-656.

² J. Exp. Med. 96: 99-106, 1952.

ENTEROVIRUSES IN ALASKA

SULKIN: Yes. In the report³ describing the immunologic classification of polioviruses into three distinct types it was indicated that they did not share a common antigen. However, other studies, such as that just referred to by Sabin, would indicate that common antigens do exist. A soluble complement-fixing antigen crosses in the CF test with heterotypic polio-myelitis antibodies.

METCALF: There are serum neutralization tests. I wonder if there is any danger of a crossing here which you might miss interpreting?

REINHARD: They were remarkably specific; however, in a population cross section study like this, in the adult group, where antibodies against all three types are present, it would be difficult to unscramble the Type antibody reactions. But note, however, the early prevalence of Type 2 reactions and the later rise of Types 1 and 3 antibodies. The peak prevalence of Type 3 antibody was hinted around twenty some years of age. Type 1 was most prevalent at age twenty or so, as I remember. Of course, the peak prevalence of Type 2 antibodies was in children.

SULKIN: Isn't this quite similar to the situation reported several years ago by Hektoen and Boor⁴ when they studied simultaneous multiple immunization of rabbits with a variety of antigens? I recall there was a remarkable response to a blunderbuss vaccine containing as many as 35 antigens, although some "crowding out" effect was observed, that is, failure of antibody to develop to one or more antigens included in the vaccine. This may account, at least in part, for the observation to which you refer.

REINHARD: Yes.

MARROW: I should like to make one observation as a physician;

³ National Foundation Committee, 1951. *Am. J. Hyg.* 54: 191-204.

⁴ *J. Infect. Dis.* 48: 588-594, 1931.

REINHARD

the emphasis has been upon natives as contrasted to Caucasian groups, but in a similar cultural environment in isolated geological survey parties in military and other construction groups, there is the same phenomenon. I have been clinically associated with medicine for fifteen years in this area. The fact is that when, on the rare occasion, the Caucasian lives in the same socio-economic status as the native, the pattern is very similar to that you describe.

REINHARD: Yes, right. And if you compare, say, the Alaska natives with the residents of Cairo, the situation is quite similar.

CAMPBELL: I want to bring up one question relative to the persistence of virus in relation to organic composition or the amount of organic material. I was just wondering whether this is actually a persistence or perhaps a growing; is it static or dynamic?

REINHARD: The organism is static. These were viability tests.

CAMPBELL: There is something in the high organic environment that stabilizes it.

REINHARD: Evidently. We could look back, for instance, to the protective action of colloids. In organic contamination by sewage, you probably have a few cations. Another factor, too, Dr. Campbell, is the fact that particularly in sewage, where there is an excessive amount of organic content, the aerobic biological activity is decreased. This is evidently a conditioning factor, for where there is a great amount of aerobic biologic activity, the virus just does not last very long, but when the environment becomes anaerobic or abiotic, then the virus may last a long while. For instance, in distilled water, the virus viability is greatly increased. Does that answer your question?

CAMPBELL: Not entirely, no. You really have to do an experiment, of course, and have those cells present.

REINHARD: The people at Sanitary Engineering Center were

ENTEROVIRUSES IN ALASKA

dealing with water in which there were actual active organisms, and they were measuring organic content, biological activity, and virus viability very closely.

WALKER: This stabilizing effect of proteins and certain ions is a fairly well known one that is used and taken advantage of regularly in the laboratory for stabilization of these and other viruses, and is used to prolong storage of viruses.

NUNGESTER: And bacteria.

REINHARD: Yes.

BERRY: Thank you, Dr. Reinhard. I suppose as moderator of this session, it is up to me to summarize everything that has been said this morning. I would not attempt it, really, but I would like to say that on the basis of this opening session, it seems rather clear that there is no evidence whatsoever of any significance that would indicate that arctic environment is particularly predisposing to human diseases of an infectious nature. There are certain unique situations that arise as a result of the environment that alter housing and in certain groups of people, the natives particularly, the diet. Their prior experience with various microbic agents, and actually these would apply not only in the Arctic, but probably in all other environments and in all other parts of the earth, shows rather clearly that in the human being, cold may or may not be stressful. In fact, the measure of man's adaptation to the arctic environment is his ability to live with minimum stress, and I assume that with minimum stress there is a minimum change and response to bacterial and viral disease.

OPENING REMARKS ON PROBLEMS OF IMMUNIZATION IN
STRESSED ANIMALS

Dan H. Campbell

Department of Chemistry
California Institute of Technology
Pasadena, California

First I will orient you with our own interest which began about when Dr. Larry Irving was setting up a laboratory at Pt. Barrow. We agreed that something should be done on immunological research and biochemical problems. From this first investigation it became obvious that many problems were apparent and should be investigated.

One of the first things that interested me was the arctic ground squirrel whose body temperature goes down to near freezing when it hibernates in the winter. If it gets a little colder, the animal wakes up and shivers and then goes back to sleep. This is a rather fantastic situation, but it might be representative of the extremes between hypothermic and normal conditions. When the squirrel is active, he makes up for the time he sleeps in various ways, so that his metabolism is probably a little abnormal both in the summer and in the winter. We first began to study the blood patterns in normal and immunized animals to see if they would produce antibodies. The idea then was to go through the gamut of tests. Most of these have not yet been completed.

We studied antibody formation, and to some extent, persistence or fate of antigens and antibodies. Most of this work has never been published. One of the first interesting problems which impressed me was that in the winter time, the squirrel's blood didn't clot. At first this was a nuisance, because we wanted the blood to clot for serum studies. This was significant in that as the body temperature decreased, the clotting time increased, and in the hibernating animal, there was practically no clot formation. Back at the

CAMPBELL

California Institute of Technology, Dr. Irving and I continued this work using rabbits. Dr. Trapani and Dr. Sutherland joined our team there.

At the California Institute, we found that when we kept the rabbits at about -20°C , their body temperature didn't go down, and therefore, one couldn't call them hypothermic animals. In order to keep rabbits alive in a "naked" state, their hair must be removed slowly. In our experiment, a strip the width of a safety razor blade was removed about every three or four days over a period of weeks until the rabbit was shaved. If the rabbit was previously conditioned to -20°C for about two weeks, he would survive and live happily at -20°C , while an unconditioned shaved rabbit would die within 24 to 28 hours. We studied these conditioned rabbits for antibody formation, half-life of antigen, and particularly the half-life of antibodies.

While in the Arctic, we also carried out a study of blood types, including Rh, in several Eskimo villages. Following this, some preliminary studies were made on lemmings. They turned out to be very poor antibody formers, which indicates for the first time that the metabolic state of the animal plays a very important role in immune mechanisms. In the cold animal stored in a cold box, antibody disappeared very rapidly, which is probably due to the rapid protein turnover. In a normal rabbit, this process was about 1/3 as fast.

If we put antigen in the arctic ground squirrels about the beginning of hibernation, it would be there at the end of hibernation just before they became active, and the same thing was true with passively transferred antibody. If either antigen or antibody was injected during the summertime when they were active, it would disappear very rapidly. Rabbits were active in the cold and their metabolism was high; under these conditions the protein turned over at a very fast rate. When antibody was injected, it rapidly disappeared. Antigen behaves in somewhat the same way, although little study has been made on this aspect as yet.

These experiments brought to mind some survival studies in Sonoma Pass that I heard about in which there occurred a very

IMMUNIZATION OF STRESSED ANIMALS

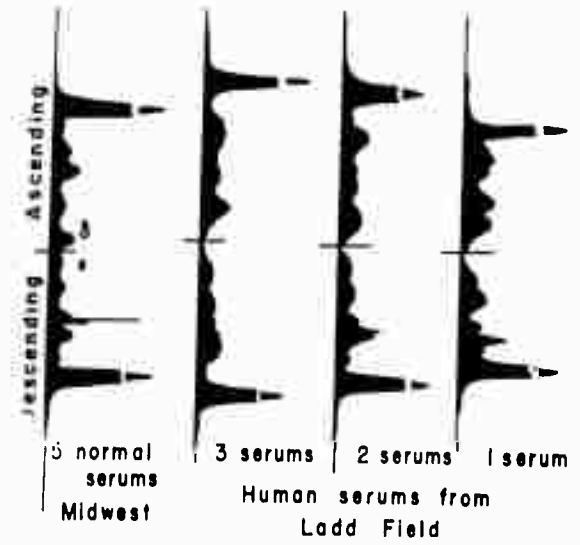


Figure 1. Electrophoretic patterns of serums from active (normal) and hibernating Arctic squirrels.

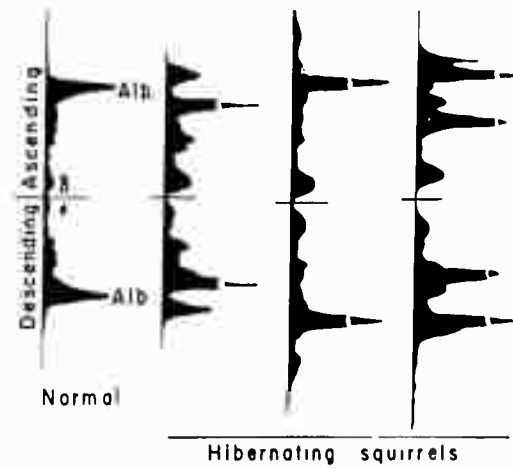


Figure 2. Electrophoretic patterns of human serums obtained during late winter from Chicago area and Fairbanks area.

CAMPBELL

high incidence of respiratory diseases among the Marines in spite of immunization. We suspect that lack of resistance was due to the rapid metabolic turnover of Ab protein.

Shown in Figure 1 is a regular electrophoretic pattern of awake and hibernating squirrels. The hibernating squirrels show a higher amount of protein than the awake squirrels which may reflect a loss of blood volume during hibernation. Note the fast-moving component which developed in a lot of the hibernating squirrels. The fibrinogen, if it is there, would probably be in the beta component. It is very difficult to interpret these data because of the extreme abnormality of the physiological state of the animal.

Figure 2 shows a comparison of serums taken in January from healthy volunteers at Ladd Field in Alaska. A peculiar thing was that the beta anomaly, usually occurring in the descending boundary of practically all normal human serums in the temperate zones, was absent. I'm not sure that this is significant, but this beta boundary disturbance is in some way associated with lipoproteins. In the summer time most serums showed this component. In the first studies, it was concluded that serums from these men did have a higher clotting time in winter than in the summer. I never saw the actual data which should be more carefully studied.

Figure 3 presents data on the retention of antigen in the livers of normal and immunized rabbits. I have included it to show that antigen in the normal rabbit will persist for a long period of time in the liver, and can be detected up to 350 days after the last injection, thus making it a baseline. Recent work by Jerislav indicates that the antigen persists a lot longer in hibernating squirrels.

Experiments designed to determine if high altitude and low temperature had any effect on experimental asthma in guinea pigs were conducted by Dr. Heimlich and Dr. Trapani at the White Mountain laboratory. The results indicated that if guinea pigs were sensitized at room temperature and sea level, they were quite resistant to challenge. Subsequent studies suggested that this resistance was apparently a stress phenomenon, and if the guinea pigs were allowed to adapt for a few weeks, they reacted like

IMMUNIZATION OF STRESSED ANIMALS

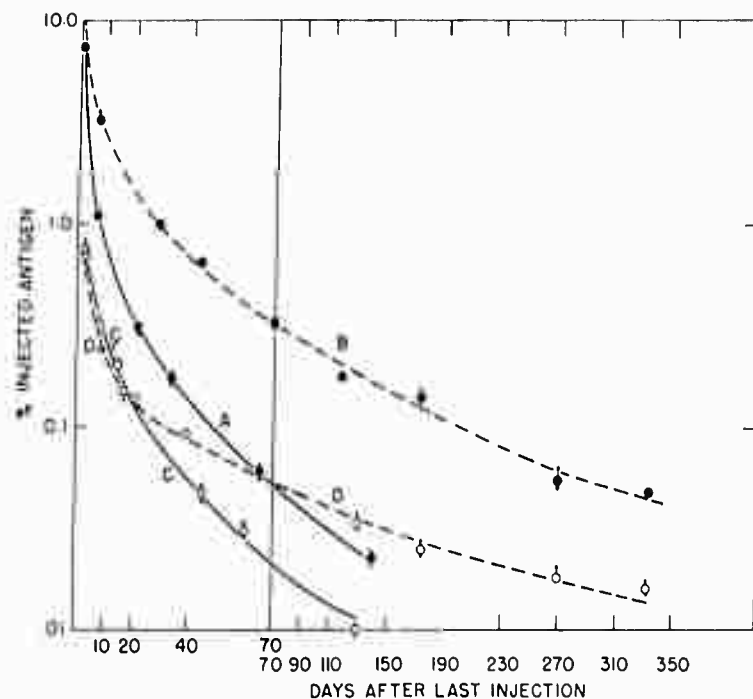


Figure 3. Semilog plot of retention in perfused liver tissue of single injection of 50 mg of S^{35} BSA (A); single injection of 50 mg S^{35} KLH (B); 9 injections of 10 mg each of S^{35} BSA (C); 9 injections of 10 mg each of S^{35} KLH (D). Each point represents average value for 3-5 rabbits. Center of circles indicates mean of distribution, indicated by the arrows. (From Garvey and Campbell, *Jour. Exp. Med.*, 105, 361, 1957.)

animals at a higher temperature and at sea level. Apparently the answer involved hypersecretion of corticosteroids.

There are many practical problems dealing with the relation of cold stress of adaptation to immune mechanisms. Many factors other than metabolism must be involved. For example, skin tests may depend to some extent on the state of peripheral capillary circulation, and immune responses in general will depend upon the previous history of the subject. In finishing, I wish to emphasize the importance of Dr. Viereck's statement; namely, that the physiological state of an animal must be determined and not assumed. Furthermore, there is a great difference between stress, adaptation, and normal states.

CAMPBELL

DISCUSSION

SULKIN: In the antigen decay experiment with the Arctic ground squirrel, I wonder if you have attempted such an experiment at a time when the animal does not ordinarily go into hibernation? In other words, have you measured antigen at another season of the year, such as the summer time?

CAMPBELL: Yes, even while they are active both antigen and antibodies disappear very rapidly in the summer time. It is a problem because squirrels do become active a while before they come out of their burrows, so actually you have to dig them out. We did have an artificial setup at Point Barrow, finally. It took us about two years to learn how to get them to hibernate. The first two years they all died; the next year somebody got some ambition and hauled down a few tons of sand from the Mead River where these squirrels live. The sand was put in a wire enclosure so the squirrels couldn't get out, and we could go out to the pen and dig them up. They become active for a couple of weeks before they come out of hibernation.

MONCRIEF: Does the inability to form antibody also imply an increased destruction of antigen?

CAMPBELL: I can't answer that because they go hand in hand. Now, in the production of antibody, antigen is destroyed. This is a fact. Now, whether antigen has to be destroyed, I don't know. Supposing it was not destroyed. If it is not broken down, it is not antigenic. We think that antigen is broken down into particles about the size of templates, and that antibody formation is just modified biosynthesis of gamma globulin by the RNA, because it is always associated with the soluble RNA; we know the soluble RNA does turn over during protein synthesis, and the more protein being synthesized, and the more active the cell is, the greater the rate of this turnover. Well, when it breaks down and turns over, then this template, or some of it, may be lost. This is a reflection of the rapid protein synthesis, and some of these fragments are always being secreted

IMMUNIZATION OF STRESSED ANIMALS

by the cell. There is a possibility, if antigen acted as a template, that it may momentarily be associated with the antibody, but actually would immediately disassociate, or disassociate soon after it got out of the cell, probably inside it. There is no question but that antigen breakdown and loss is associated with antibody formation.

MONCRIEF: Not necessarily the other way around?

CAMPBELL: No.

MONCRIEF: Do you know anything about the diet of the hibernating animal with respect to protein, carbohydrate, and fat composition that these people on Ladd Field worked with when they measured clotting time?

CAMPBELL: The hibernating animal, of course, isn't eating.

MONCRIEF: Prior to his going into hibernation.

CAMPBELL: He is in pretty good shape under natural conditions. I don't know what all they do eat, besides berries and roots, and so on.

MONCRIEF: The only reason I ask, is that a very peculiar observation came up about a year ago; Walter Blum was putting patients on starvation diets. These patients were placed on a completely carbohydrate-free diet, nothing but fat and protein; he drew blood samples from these patients and placed them in the freezing portion of the ice box, and a few weeks later when I happened to be visiting him, he took samples out to show them to me. He pulled about twenty samples of blood out of the refrigerator, six of which were from patients on this diet. The other fourteen were frozen solid, but the six on this diet were still completely liquid. He later analyzed these for everything he could consider possible and found nothing to be abnormal in the blood except an elevation of the non-esterified fatty acids. Even serum osmolarity was the same.

CAMPBELL: I forgot to mention, these sera that don't clot,

CAMPBELL

and even the rabbit sera that clot very poorly, are very low in complement. Complement has always been associated with clotting. I don't know just what the connection is, but the French used to consider prothrombin. Well, that turned out to be not true, but these hibernating squirrels have practically no complement, and in rabbits' sera that have been stored for a while, the complement goes down; it goes along with the blood clotting. It would be interesting to study the complement titer which might go down very rapidly in some of these patients where the blood clotting goes down after hypothermia.

BLAIR: Yes, this occurs only at fairly deep levels of hypothermia, and I think it is important to bring this out. It has been traced to reduction of platelets, and this is probably due to trapping in the capillaries. The periods of hypothermia at this level are so short that it is quite unlikely that anything happens to the fundamental mechanisms that involve the clotting. Whether there is any actual alteration in the protein response is probably unlikely for these short periods.

CAMPBELL: But even if the complement was reduced for a short period, it might play a role.

TRAPANI: Are any of these serum changes detectable before hibernation, or just following hibernation?

CAMPBELL: This is the problem, of course, and, let's see, maybe Dr. Tunevall could tell us about this work in Sweden on the polypeptide from the brown fat. I think they have been working on it in the porcupine. This is the problem that really intrigues me. The brown fat evolves during hibernation; and even the shaved rabbits will begin to show a little brown fat. This has always been associated with hibernation. If you could isolate a polypeptide, it would be the perfect anesthetic. This was realized, I think, quite a few years ago.

ENVIRONMENTAL EXTREMES AND ENDOCRINE RELATIONSHIPS IN ANTIBODY FORMATION¹

Ignatius L. Trapani

Department of Experimental Immunology
National Jewish Hospital
Denver 6, Colorado

ABSTRACT

This paper concerns antibody production and decay in animals exposed to environmental extremes of low temperature (-15°C) or high altitude (12,500 ft. and 14,500 ft.), or in which an imbalance in endocrine activity has been produced. It soon became apparent from our earlier studies that it was not possible to investigate the effect of environmental stress without implicating physiological alterations which might occur and thus influence the synthesis and metabolism of antibody. Our studies were extended to include animals which were in endocrine imbalance in an attempt to isolate one of several physiological factors which might be altered under conditions of stress. The study of these physiological factors, their inter-relationships, and their influence on antibody synthesis and decay is expressed in the term immunophysiology. The study of adrenalectomized, thyroxin-treated, and surgically thyroidectomized animals helps to explain the immune response of cold-exposed animals which exhibit an increased thyroid activity. The immune response can be thought of as being composed of two processes; antibody production and antibody decay, occurring simultaneously, but not necessarily at the same rate. For example, a net increase in circulating antibody might arise from (a) an unchanged production associated with a decreased rate of decay; (b) an increased production associated with a decreased rate of decay; or (c) a decreased production associated with a marked decrease in decay rate.

The discussion I wish to present concerns antibody production and decay in animals exposed to environmental extremes of low temperature (-15°C) or high altitude (12,500 ft. and 14,150 ft.), or in which an imbalance in endocrine activity has been produced (Trapani, 1957, 1960, 1961; Trapani and Campbell, 1959; Trapani, Lein, and Campbell, 1959a and 1959b; Trapani and Jordan, 1962).

¹ These studies were aided by Contract Nonr 3545(00) (NR 102-573) between the Office of Naval Research, Department of the Navy, and the National Jewish Hospital at Denver.

TRAPANI

The experimental animal was the rabbit; the antigen-antibody system used was bovine serum albumin (BSA) and anti-BSA precipitins. The low temperature studies were done in an especially constructed cold box having a capacity of 10 rabbits. Adequate provision was made for lighting and fresh air without drafts, and the animals were kept on wire mesh in individual cages. Water was changed four times a day because of freezing and standard food was provided *ad libitum*. Animals exposed to high altitude were maintained at the Barcroft Laboratory (altitude =12,500 ft.) of the White Mountain High Altitude Station in California, or, in more recent studies, at the Summit Laboratory (altitude =14,150 ft.) of the Inter-University High Altitude Laboratory, Mount Evans, Colorado.

Much of the work discussed here was done in collaboration with Professor Dan H. Campbell at the California Institute of Technology.

It became apparent from our earlier studies on the immune response that it was not possible to investigate the effect of environmental stress without implicating physiological alterations which might occur and thus influence the synthesis and metabolism of antibody. Our initial studies on the effect of low environmental temperature and high altitude were extended to include experiments on animals which were in endocrine imbalance in an attempt to isolate one of several physiological factors which might be altered under conditions of stress. The study of these physiological factors, their inter-relationships, and their influence on antibody synthesis and decay is expressed in the term immunophysiology.

The purpose of my discussion is not necessarily oriented toward the elucidation of all of the problems relating to antibody formation, but rather to emphasize some of its complexities and some of the secondary factors which influence the immune response. The topic of interest, for the moment, is not concerned with speculations relating to cellular mechanisms *per se* which may be responsible for, or may participate in, the synthesis of a particular protein by antibody forming cells. Rather, I will attempt to elucidate the role of certain physiological factors which influence the basic synthetic mechanisms involved in our test system.

Buried in the literature of the exploits of people exposed, either

ANTIBODY FORMATION

by design or accident, to environmental extremes of low temperature or high altitude are accounts of changes in resistance to infection and disease. However, little quantitative experimental work on the immune response has been done under controlled environmental conditions. Some of our first studies were those utilizing rabbits acclimatized to an environmental temperature of -15°C , or else exposed to an altitude of 12,500 ft. at the Barcroft Laboratory of the White Mountain High Altitude Station in California.

A first approach to the investigation of the immune response was the study of protein turnover, by passive immunization techniques. In this procedure, homologous gamma globulin containing specific antibody is injected intravenously into recipient animals. The animals are bled periodically, and the concentration of serum antibody estimated by quantitative micro-precipitin analysis (Lanni, Dillon, and Beard, 1950). The results are plotted semi-logarithmically as the percentage of the injected dose remaining in the circulation versus time. The slope of the linear portion of the curve (assumed to be steady state loss of antibody) is calculated by the method of least squares, extrapolated back to zero time, and the half-life of the injected antibody calculated from that point.

The half-life of passively administered homologous antibody for controls, cold-exposed, and high-altitude adapted animals was: 4.7 ± 0.2 , 3.4 ± 0.2 , and 4.5 ± 0.2 days, respectively. The cold exposed animals have a significantly increased rate of antibody turnover, while those at high altitude do not.

Rabbits exposed to -15°C for 10 weeks and clipped of hair during the latter half of that period were actively immunized by the subcutaneous injection of BSA (10 mg per kg body weight) in Freund's adjuvant. Levels of circulating antibody were followed for a period of 52 weeks (Fig. 1). Rabbits maintained at room temperature and treated in the same manner were used as controls.

The level of circulating antibody in the cold exposed group increased at a slower rate than in the control group and approached control levels at approximately 14 weeks. There was then a decline in both groups, but at different rates, throughout the remainder of the experimental period, so that by the end of 52 weeks the level

TRAPANI

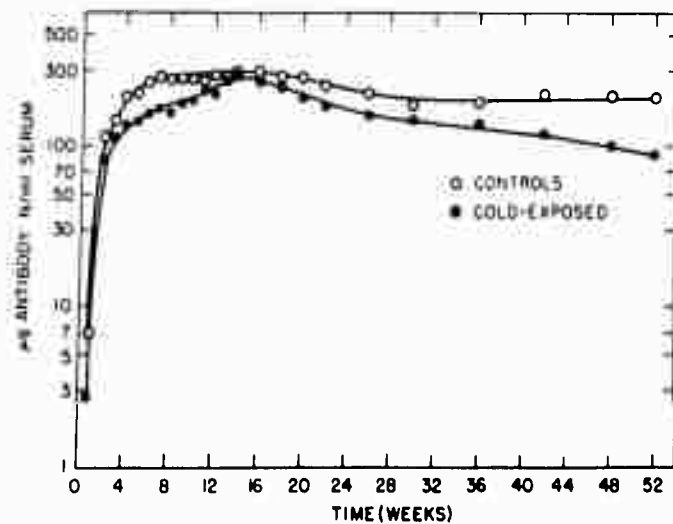


Figure 1. Circulating antibody levels in rabbits immunized with BSA plus Freund's adjuvant (10 mg BSA per kg body wt) at time zero. The cold exposed group, kept at an environmental temperature of -15°C , was progressively clipped during the first 8 weeks and immunized after a 10-week exposure.

of circulating antibody in the cold exposed group was approximately 50 per cent of that in the control group.

If these data alone are considered, it would appear that the cold exposed animal does not synthesize antibody as well as the non-exposed animal. However, it must be remembered that curves of this type represent not only antibody production, but also antibody decay. Thus, part of the difference may be attributed to an increase in protein degradation, as inferred from passive antibody decay studies.

Since cold exposed animals have been shown to have increased thyroid activity, it seemed important to investigate the activity of the thyroid gland on the immune response. Studies were based on the a priori assumption that hyperactivity of the thyroid contributed to the immune response of cold exposed animals. The interesting and complicating fact is that even though the assumption may be valid, the experimental observations did not answer completely our questions.

ANTIBODY FORMATION

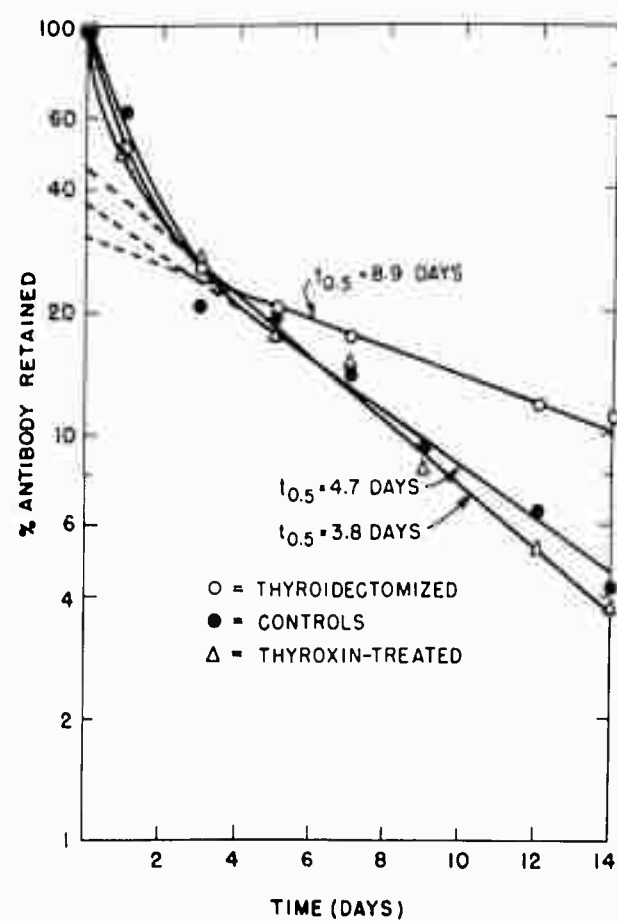


Figure 2. Passive antibody decay in surgically thyroidectomized, thyroxin-treated and untreated control rabbits.

TRAPANI

A study was made of passive and active immunization in rabbits which were either surgically thyroidectomized or treated with thyroxin. The half-life of passively administered antibody in thyroidectomized, thyroxin-treated, and controls (Fig. 2) was: 8.9 ± 0.4 , 3.8 ± 0.2 , and 4.7 ± 0.2 days, respectively.

Similarly prepared groups of rabbits were actively immunized with BSA (10 mg per kg body weight) in Freund's adjuvant. Figure 3 displays the data obtained from the experiment. The circulating antibody level in the thyroidectomized group rises gradually and approximates the control level at about 14 weeks after immunization. The thyroxin-treated group, on the other hand, not only had an initially higher level of circulating antibody, but also displayed measurably increased amounts of antibody 3 days after immunization. This early response was not present in either of the other groups. After the initial high level of circulating antibody in the thyroxin-treated group, there was a decline to levels below those of controls, and then a second increase. Thus, there is a similarity between hyperthyroid and cold exposed animals in terms of an increased rate of protein turnover. However, the net immune response after active immunization shows little similarity between hyperthyroid and cold exposed rabbits.

Since experiments concerned with thyroid activity did not clarify the results obtained with cold exposed animals, we next investigated the role of the adrenal gland on the immune response. One reason for this approach was the observation that high-altitude acclimatized rabbits showed an increased immune response, and animals under these conditions exhibit an increased adrenal activity.

Figure 4 shows the results of active immunization of rabbits acclimatized to 14,150 ft. at the Summit Laboratory of the Inter-University High Altitude Laboratory at Mount Evans, Colorado. Immunization was accomplished with a single intravenous injection of 10 mg BSA per kg body weight. The animals were bled periodically for the next 5 weeks and then returned to Denver (altitude = 5,280 ft.) where further samples were taken. After circulating antibody had reached low levels, the animals were given a secondary intravenous challenge with 10 mg BSA per kg body weight. Sixteen weeks later, a third immunization dose was given in the same

ANTIBODY FORMATION

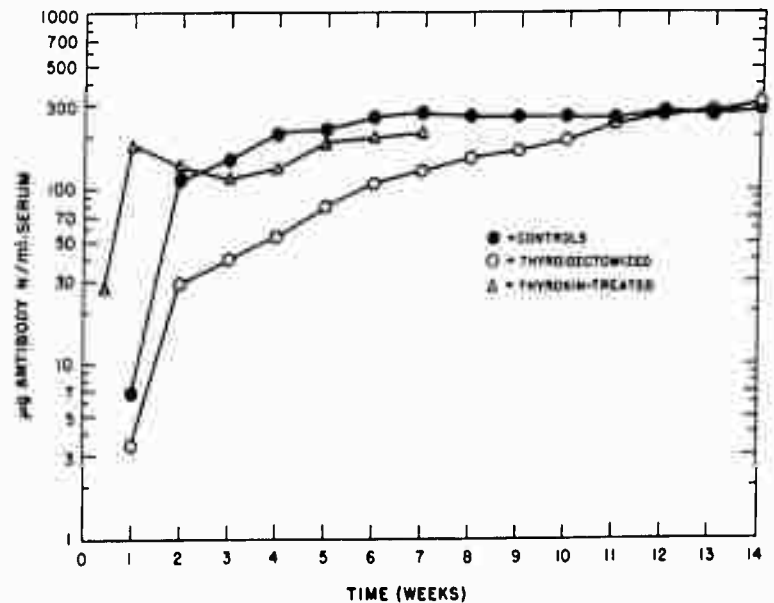


Figure 3. Circulating antibody levels in surgically thyroidectomized, thyroxine-treated, and untreated control rabbits immunized with BSA plus Freund's adjuvant (10 mg BSA per kg body wt) at time zero.

manner. Maximum utilization of the experiment could be had in this way, and information relative to the time course of re-adaption to the lower altitude obtained.

After primary injection, the animals at high altitude had levels of circulating antibody approximately 60 per cent higher than controls, and were significantly different. After the secondary challenge, the group previously exposed to high altitude had levels of circulating antibody approximately 35 per cent higher than controls, even though they had been residing at the lower altitude for about six weeks. A third immunization, 22 weeks after descent to lower altitude, showed no difference between the two groups. In this experiment, the time course of response to the antigenic stimulus was similar for both groups. The maximums reached, however, by the high-altitude group were greater both while at the mountain and 6 weeks after returning to the lower altitude.

TRAPANI

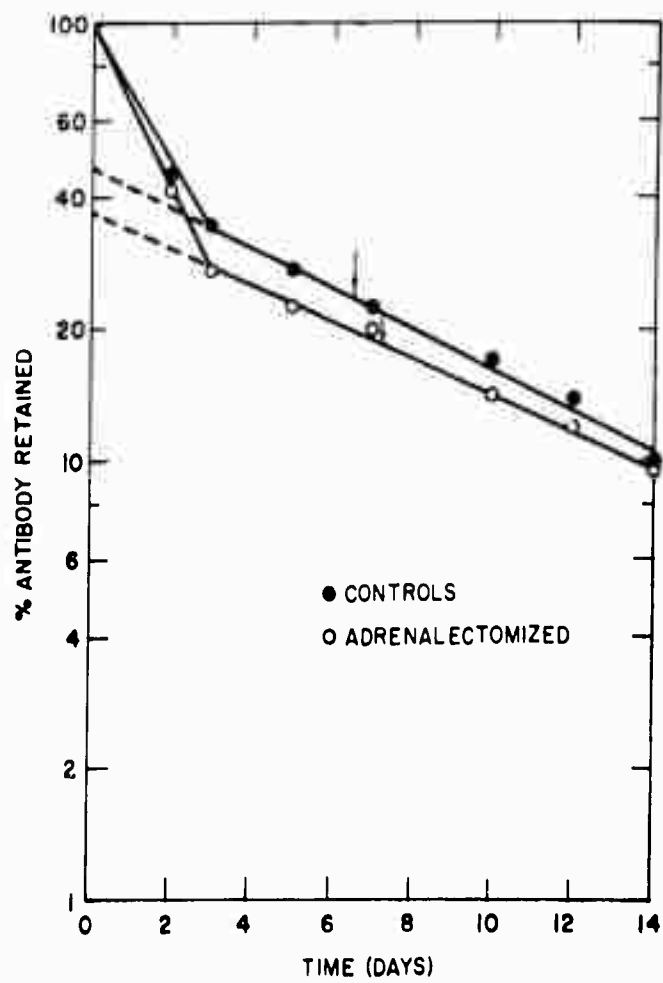


Figure 4. Passive antibody decay in bilaterally adrenalectomized and control rabbits.

ANTIBODY FORMATION

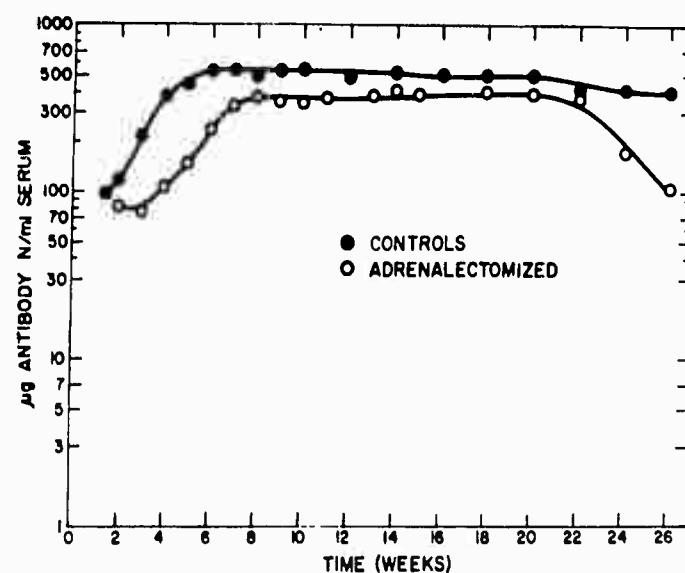


Figure 5. Circulating antibody levels in bilaterally adrenalectomized and control rabbits immunized with BSA and Freund's adjuvant (10 mg BSA per kg body wt) at time zero.

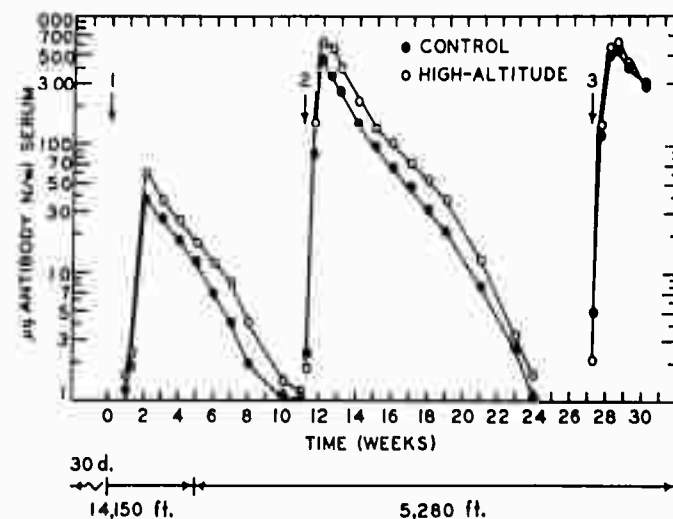


Figure 6. Circulating antibody levels in rabbits adapted to high altitude and immunized with BSA (10 mg per kg body wt) intravenously. 1 indicates the primary injection, and 2 and 3 indicate the second and third challenge, respectively.

TRAPANI

The next point of information I wish to present is that of the immune response of adrenalectomized animals. Even though there is an abundance of literature dealing with the effect of the adrenal steroids on various aspects of the immune response, little has been done utilizing extirpation experiments. If the reaction of the animal, deprived of its endogenous source of hormone, can be established, then the activity of various available steroid preparations might be more accurately assessed.

Rabbits were bilaterally adrenalectomized in a one-stage operation (Zak, Good, and Good, 1957) using a ventral mid-line approach. They were maintained on 1 per cent saline for drinking water with free access to food, and allowed to recover for 3 to 4 weeks before being used in the experiments. Passive decay of antibody (Fig. 5) in adrenalectomized rabbits was not significantly different from control animals. Active immunization (Fig. 6) shows an apparent net decrease in the immune response of adrenalectomized rabbits.

It has been reported that adrenal steroids depress the immune response. The data presented here, however, indicate that adrenalectomy results in a decreased immune response. These apparently contradictory results present a paradox, and point to the complexity of experiments of this kind.

DISCUSSION

The immune response can be thought of as composed of two processes (antibody production and antibody decay) occurring simultaneously, but not necessarily at the same rate. It is possible to measure the decay rate of passively administered homologous antibody; however, what is measured in the actively immunized animal is the net result of antibody production and decay. It is conceivable, therefore, that an increase in antibody production might be offset by an increase in antibody decay so that the net level of circulating antibody measured is apparently unchanged.

ANTIBODY FORMATION

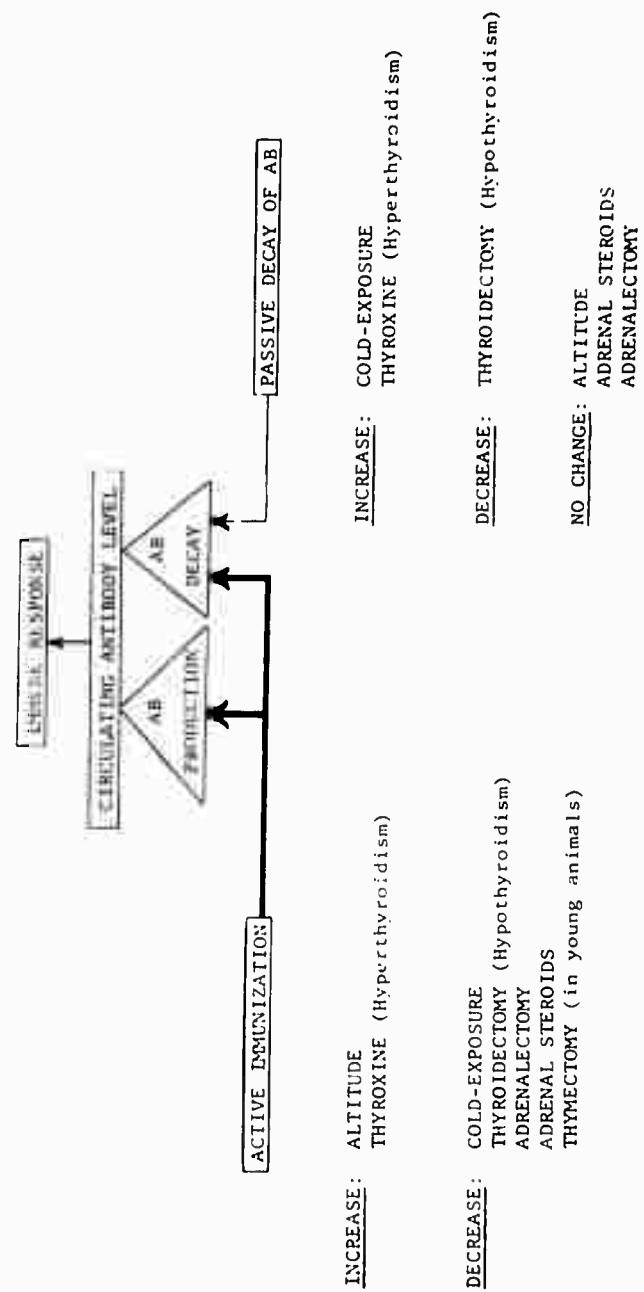


Figure 7. Environmental and endocrine factors which may influence the immune response.

TRAPANI

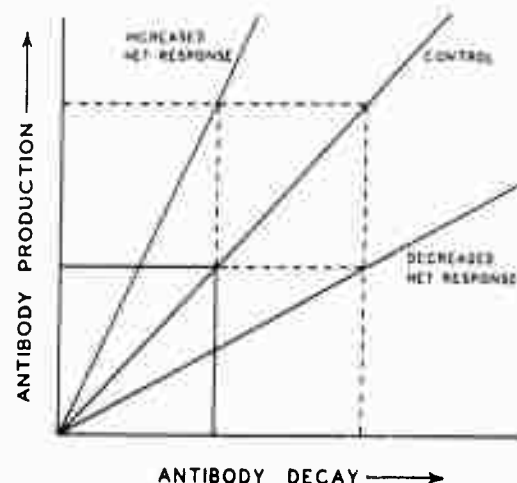


Figure 8. Relationship of antibody production and antibody decay to the net immune response.

Some of the complexities of the physiological relationships which might pertain to the immune response of animals exposed to environmental extremes or endocrine imbalance can be derived from Figure 7. For example, cold exposed animals exhibit a decreased immune response, as measured by circulating precipitating antibody. This may be the result of a) an unchanged rate of production associated with the increased rate of decay, b) a decreased rate of production associated with an increased rate of decay, or c) an increased rate of production which is not of sufficient magnitude to offset the increased rate of decay.

Figure 8 presents a theoretical relationship between antibody production and decay. If production and decay are "balanced" against each other, we then arrive at the line labeled "control". If we now manipulate the animals' physiology so that antibody production or decay is altered, it is possible to arrive at a net response which is either increased or decreased. If production and decay are altered to the same degree and in the same direction, the net response could still fall on the control line, and the observation would be no net change in response. As a consequence, the actual alteration would be obscured in this test system, and other avenues would have to be explored to arrive at a more definitive answer.

ANTIBODY FORMATION

The persistence of antigen (Garvey and Campbell, 1956) and its degradation in the host animal may also be altered under conditions of environmental extremes or endocrine imbalance. Recent studies of antigen disappearance in hibernating ground squirrels (Jaroslow and Smith, 1961) have shown that there was no detectable disappearance of antigen during 14 days of hibernation. After arousal, however, the induction period for antibody formation was shorter than in non-hibernating animals. It appears that this response is a reflection of the physiological state of the animal.

Even though certain physiological changes might be implicated a priori, it is difficult to attribute the net immune response to only one factor. It appears essential to consider the immunophysiological inter-relationships and the functioning of the animal as an integrated unit in host-parasite interactions.

SUMMARY

Deductions made for host-environmental interactions can often be derived by more than one pathway involving secondary physiological factors which may affect resistance to disease, antibody formation, and antibody decay. The immune response of animals exposed to environmental extremes or endocrine imbalance must be interpreted in the light of available knowledge concerning the functioning of the animal as an integrated unit.

TRAPANI

LITERATURE CITED

1. Jaroslow, B. N., and D. E. Smith. 1961. Antigen disappearance in hibernating ground squirrels. *Science* 134: 734-735.
2. Lanni, F., M. L. Dillon, and J. W. Beard. 1950. Determination of small quantities of nitrogen in serological precipitates and other biological fluids. *Proc. Soc. Exp. Biol. Med.* 74: 4-7.
3. Trapani, I. L. 1957. Antibody decay in cold exposed rabbits. (Abstr.) *Federation Proc.* 16: 436.
4. Trapani, I. L., and D. H. Campbell. 1959. Passive antibody decay in rabbits under cold or altitude stress. *J. Appl. Physiol.* 14: 424-426.
5. Trapani, I. L., A. Lein, and D. H. Campbell. 1959. The effect of thyroidectomy and thyroxin treatment on the immune response of rabbits. (Abstr.) *Federation Proc.* 18: 161.
6. Trapani, I. L., A. Lein, and D. H. Campbell. 1959. Passive antibody decay in thyroidectomized rabbits. *Nature* 183: 982-983.
7. Trapani, I. L. 1960. Cold exposure and the immune response. In *International Symposium on Cold Acclimatization*, Buenos Aires, Argentina, August, 1959. *Federation Proc.* 19: Suppl. 5, 109-114.
8. Trapani, I. L. 1961. The immune response in adrenalectomized rabbits. (Abstr.) *Federation Proc.* 20: 23.
9. Trapani, I. L., and R. T. Jordan. 1962. Antibody formation in rabbits adapted to high altitude. (Abstr.) *Federation Proc.* 21: 25.
10. Zak, S. J., R. H. Good, and R. A. Good. 1957. A technique for one-stage bilateral adrenalectomy in the rabbit. *Nature* 179: 100-102.

ANTIBODY FORMATION

DISCUSSION

BERRY: I am very much interested in this report, Dr. Trapani, and would like to point out some of the effects of exposure to a simulated altitude higher than yours; that is, 20,000 feet. One of the things that we have noticed is that mice show an increase in urinary nitrogen excretion. By inference, this would suggest that protein catabolism is increased. If one attains an elevation in nitrogen excretion, this says that the animal is breaking protein down fast. We find it in mice that have been exposed to simulated 20,000 feet for as long as a month, and these animals are about as fully acclimated, as judged by their general metabolism responses as any animals that we have studied. We have kept them for as long as three months, and we can find no difference in animals kept for three months at simulated 20,000 feet than those kept for three or four weeks. We can also get this elevated urinary nitrogen excretion in animals that have been exposed for one day at 1000 feet; and I don't know what this means. This is a very bewildering thing to us.

I wonder if in any of these rabbits urinary nitrogen excretion was determined?

TRAPANI: No, I would suspect that almost any stress imposed on the animal would change its urinary nitrogen excretion. If an animal is put in a cage which is wired with electrical current which goes on and off at intervals, I imagine this sort of a stress might influence nitrogen excretion. Experiments that you just mentioned are somewhat similar to those done by Mefford and Hale¹ in which the metabolic interrelationships of cold, heat, and altitude were studied. One of their points, of course, is that there is an increase in nitrogen excretion.

BERRY: What produces the increase in nitrogen excretion,

¹ Am. J. Physiol. 193: 443, 1958.

TRAPANI

metabolically?

TRAPANI: Off hand, I don't know.

CAMPBELL: Doesn't the salt balance play an important part?

WALKER: Do these animals lose weight, or eat more, Dr. Berry?

BERRY: They do not lose weight. They are in pretty good metabolic balance as judged by weight.

WALKER: They take in more protein to keep that balance?

BERRY: Yes, but these are complicated relationships.

TRAPANI: The main purpose here, for me, was to point out how complex this really is. These experiments are really not sufficient yet to make a complete story by any means, and the next step planned is to study the immune response of adrenal-ectomized animals for a considerable period, to elucidate the relationship between the thyroid and the adrenal. Despite all of your excellent experiments utilizing the cold and high altitude chambers, I personally like mountain top laboratories better, since I feel that chambers do offer certain disadvantages.

MONCRIEF: Do your rabbits that you shave still shiver?

TRAPANI: I never saw them shiver.

MONCRIEF: Are they acclimatized to the cold?

TRAPANI: They seem to be. Their body temperature was not much different from room temperature controls.

MONCRIEF: What evidence do you have of increased metabolism of rabbits?

TRAPANI: The metabolic rate, based on oxygen consumption measurements done by open circuit metabolimetry was approxi-

ANTIBODY FORMATION

mately twice that of room temperature controls. Serum protein concentration also increases. This might be an interesting point also to mention. Animals at high altitude have an increased serum protein concentration and a decreased plasma volume. Animals in the cold also have an increased serum protein concentration, and an increased plasma volume. Their plasma volume was measured by Evans blue disappearance and also from the passive antibody decay studies. In the latter case, the amount of antibody injected and the initial antibody concentration in the serum are used to calculate "plasma volume", or perhaps we should call it "antibody space", as compared to Evans blue space.

TUNEVALL: Was the decreased plasma volume at high altitude simply a result of an adaptive polycythemia?

TRAPANI: Yes. From the information just given, it was calculated that animals at high altitude had a total mass of circulating proteins the same as controls, while animals in cold had an increased mass of circulating protein. More specifically, if one makes the proper corrections, the increase in the immune response of animals at high altitude is greater than that which might be expected from a decreased plasma volume.

PREVITE: Have you ever done any experiments in a shorter period of time? For example, have you ever measured antibody titers in response to BSA prior to one week, or isn't that a sufficient amount of time to get a response?

TRAPANI: It's very difficult to detect antibodies before seven days or so.

PREVITE: Have you ever previously immunized your rabbits, waited a sufficient amount of time, and then put them at -15° C?

TRAPANI: Not in the cold. We did one experiment with rabbits at high altitude similar to what you are thinking about. The first year we took animals up to White Mountain in Cali-

TRAPANI

fornia. Some were immunized in Pasadena before going up, some were immunized when they first got there, some were immunized after seven days, some after a month, and some were just brought up there for a month, then immunized and returned to Pasadena. The only group which seemed to give a fairly clear picture so that we could say, "Well, let's study this one because it's a little simpler," was the group which was brought to a high altitude, adapted for thirty days and then immunized, and the immune response studied while the animals were kept at high altitude. The variation of response in the other groups was too great to merit further study at the time.

PREVITE: I am wondering what would be the antibody response to cold stress within the first few days if one used a rabbit with a known titer? Would you expect antibody titers to decrease?

TRAPANI: By inference, I would say antibody levels may go down; these are given levels of circulating antibody. When the animal is put into the cold, the metabolic rate is increased to overcome the heat load; antibody levels might then decrease as a consequence of an increased turnover rate.

PREVITE: It seems that most of us agree that normally we are warmly clothed when we are outdoors in cold weather. Maybe one is only accidentally going to be stressed by the cold environment. Perhaps it is most important, in analysis of the effects of cold exposure on infectious diseases, to determine the response to this chance exposure.

TRAPANI: I would like to make one more comment that Dr. Berry brought to mind. I think the antigen must be considered not only in terms of its persistence, but also in regard to its chemical nature. The system I used was a soluble one of BSA and anti-BSA. If one uses a viral or bacterial system, these different kinds of antigen might be handled differently by the animals under these situations. Dr. Berry has shown that in animals subjected to high altitudes, resistance to bacterial infection is decreased, whereas resistance to viral infection is

ANTIBODY FORMATION

increased. At present, I am doing an experiment in collaboration with Dr. M. L. Cohn in which Guinea pigs kept at -4°C at the Denver altitude or on top of Mount Evans, are infected with Mycobacterium tuberculosis. We just finished infecting our animals by inhalation techniques last week, and whether or not the results will come out similar to yours won't be known for another month or so.

QUALITATIVE AND QUANTITATIVE ASPECTS OF THE IMMUNE RESPONSE UNDER CONDITIONS OF COLD EXPOSURE

William T. Northey

Department of Microbiology
Arizona State University
Tempe, Arizona

ABSTRACT

Rabbits have been exposed to lowered environmental temperatures and immunized with various protein antigens. In certain experiments, the immunization and bleeding schedules have also been changed. Serum samples from these animals, as well as a "control" group receiving an identical immunization schedule, have been analyzed by a number of immunological and immunochemical techniques. To detect any possible qualitative differences in the serum samples as a result of cold exposure, a number of analyses have been conducted. Among the techniques used are: Ouchterlony gel diffusion, immunoelectrophoresis, and starch gel electrophoreses. Quantitative differences in the serum proteins have been studied by a determination of A/G ratios and by a comparison of the serum globulins by paper electrophoresis. Precipitin titrations on rabbit sera have been conducted using the ring test, and total antibody nitrogen has been measured by the quantitative precipitin technique.

It may seem unusual to many in this group that Arizona State University, which is located in the heart of the Sonoran desert, would be an institution in which studies concerned with "cold stress" are conducted. However, for the past two years, including the months of June, July and August when the outside temperature exceeds 37.7°C almost daily, we have, through the marvels of modern day refrigeration, continued our studies into the immunological aspects of this fascinating and challenging problem.

Many observations concerning the influence of environmental temperature have been made on experimental animals and human beings as well. But the seasonal incidence of many diseases has never been explained, although it has been suggested by some investigators that environmental temperatures may influence the frequency with which a disease may occur, as well as the severity

NORTHEY

of the infection (Moragues and Pinkerton, 1944; Jungeblut et al., 1942). In numerous studies on the influence of environmental temperature on both human and experimental infection, conflicting results have been reported, even when the same infectious agents and the same animals were used. In the majority of these studies, no attempt was made to measure the antibody response quantitatively of animals under "cold stress". A notable exception to this has been work of Campbell, Trapani and Sutherland (Campbell, 1951; Sutherland et al., 1958) whose studies on the sera of cold exposed rats and rabbits has provided valuable information concerning passive antibody decay, changes in the serum proteins, and alterations in the antibody level and blood chemistry. I attempted to measure qualitatively and quantitatively the immune response of rabbits exposed to lowered environmental temperatures. To achieve a high degree of sensitivity, antigen-antibody systems of known high reactivity were used, and the results of the immunization of both "normal" and "cold exposed" rabbits studied. Through the use of strongly reacting protein antigen-antibody systems, a qualitative measure of the antibody response was made through the use of such techniques as Ouchterlony gel diffusion, immuno-electrophoresis, starch gel electrophoresis, and paper electrophoresis. Quantitative determinations included titrations of antibody levels by the conventional "ring" precipitin technique, and by the much more precise method of micro-quantitative precipitin analysis. An attempt was made to obtain quantitative results of gel diffusion studies by measuring the intensity and homogeneity of the antigen-antibody reaction in terms of the amount of precipitate formed. The objective was to determine (1) the sensitivity of the immune mechanism of animals during cold exposure versus the "normal", that is, the multiplicity of the antibody response, and (2) the "type" of antibody produced during cold exposure, that is, the affinity or avidity for its specific antigen of the antibody produced by the "cold exposed" versus "normal" animal. The specific objective of the qualitative studies was to determine the number of multiple antigen-antibody systems which can be observed in each of the groups when identical immunization schedules are followed. Immunization with the same multiple antigen system will provide information regarding the sensitivity and selectivity of the immune mechanism under conditions of the cold exposure. By studying both the qualitative and quantitative aspects of the problem, one should be able to draw certain conclusions regarding the response of the "cold exposed" ani-

IMMUNE RESPONSE IN COLD EXPOSURE

mals in terms of (1) the degree of response, for example, the total amount of antibody produced, (2) the rate of appearance of antibody, (3) the "type" of antibody produced, and (4) the influence of the period of immunization and/or cold exposure on the response of animals to major and minor antigens when immunized with a multiple antigen system.

This information may serve to elucidate the role of the "specific" factor of immunity, that is, circulating antibody during periods of cold exposure, and at the same time serve to clarify the response of the immune mechanism to immunization with multiple antigens both during and prior to cold exposure.

In all of these studies, the rabbit was the experimental animal of choice. The animals were housed in a walk-in cold room maintained at a temperature of 4°C. Prior to the beginning of the immunization schedules, the rabbits were allowed to "adapt" for a period of one week, after which they were clipped until fur remained only on the head and the extremities. New fur was periodically clipped. The animals were given food ad libitum, and water was changed frequently. Open wire cages were used to allow free movement of air. Only one rabbit was housed in each cage to prevent huddling. In most of the studies, rabbits weighing approximately 2.5 to 3.0 kg were immunized with three weekly intra-muscular injections of equal parts of antigen and Freund's adjuvant (Freund, 1947).

Blood samples were obtained via cardiac puncture, the serum separated and merthiolate added in a 1:10,000 final concentration. All of the rabbits used in these studies were carefully selected in order to control genetic variations. Litters of six or more were raised for this investigation, and each litter was divided into equal study groups of experimental and control animals. The serum samples were then subjected to a variety of immuno-chemical analyses which were designed to detect any difference in the serum proteins of the "cold exposed" versus the "non-cold exposed" rabbits.

The immune response of an animal to the administration of an infective agent is best measured in terms of the antibody formed and directed against the microorganism and its antigenic spectrum.

NORTHEY

While the original aim of this investigation was to determine whether or not differences exist in response to antigenic stimulus in "cold stressed" animals as opposed to "normal" animals following immunization with various selected microorganisms, preliminary experiments indicated that these systems were not sensitive enough, nor did they possess a high degree of specificity. Early experiments were designed to measure qualitative and quantitative differences in response to the injection of various selected microorganisms such as Salmonella typhimurium and Diplococcus pneumoniae. Analysis of the sera collected from animals challenged in this manner proved to be difficult in that as the gel diffusion technique best serves as a measure of the "soluble" antigen content of a microbial suspension, the degree of sensitivity which could be attained using microbial anti-microbial systems was inadequate to detect differences in the "cold stressed" and control animals. Because of the high degree of reactivity inherent to antigen-antibody systems involving complex soluble protein antigens, a number of systems involving these antigens were selected for investigation. As a result, the original experimental protocols were modified to include a number of new systems which are of sufficient complexity and sensitivity for a study of this type. These new studies included such protein antigens as human serum, egg albumin (Ea), bovine serum albumin (BSA), and whole bovine serum. These protein antigens, although not implicated in the usual "infective process", serve as a reliable index of the degree of sensitivity and selectivity by the antibody forming mechanisms.

METHODS

Gel Diffusion

One of the most sensitive methods of measuring the antibody response is through the use of the gel diffusion technique devised by Ouchterlony (1949). In this investigation a 1 per cent solution of Ionagar Number 2 (Consolidated Laboratories, Chicago Heights) was used to prepare the gel. The pattern was cut in the agar using a Fineberg Agar Gel Cutter Number 1802, manufactured by the

IMMUNE RESPONSE IN COLD EXPOSURE



Figure 1. Ouchterlony gel diffusion analysis of bovine serum--rabbit antiovine serum.

Shannon Scientific Company of London, and distributed in this country by Consolidated Laboratories. This pattern consists of a center well surrounded by four wells equidistant from it. These four wells were used for the various antigen dilutions. In order to approximate more closely the region of optimal proportions, four samples of antigen were tested in each case; that is, undilute, 1:2, 1:4, and 1:8. Plates were read at appropriate intervals in order to determine the time of precipitation of the first reacting components, and the results of each of the readings were recorded on master sheets designed for this purpose. Readings in all cases were taken from the antigen-antibody concentrations which gave the most definitive results. A sample plate is shown in Figure 1.

For the data obtained to be best evaluated, it became necessary to adopt a more accurate method of measurement based on statistics. The method of choice was the Mann-Whitney U test. This nonparametric test is a useful alternative to the parametric "t" test when the measurement in the research is weaker than interval scaling.

Aladjem et al., (1959) have reported that the time lapse preceding

NORTHEY

Sample	N	R-Cold	R-Normal	U *
1	9	85.500	85.500	40.500
2	9	86.500	84.500	39.500
3	9	66.000	105.000	21.000
4	9	68.500	102.500	23.500
5	9	65.500	105.500	20.500
6	9	70.500	100.500	25.500
7	9	63.000	108.000	18.000

Table I. Quantitative evaluation of precipitates in gel diffusion of sera from "normal" and "cold exposed" rabbits. *U value of Mann-Whitney Utest at 0.05 significance level = 17. Antigen-whole bovine serum.

Sample	N	R-Cold	R-Normal	U*
1	9	85.500	85.500	40.500
2	9	83.500	87.500	38.500
3	9	80.000	91.000	35.000
4	9	82.500	88.500	37.500
5	9	68.500	102.500	23.500
6	9	69.500	101.500	24.500
7	9	85.000	85.500	40.500

Table II. Quantitative evaluation of precipitates in gel diffusion of sera from "normal" and "cold exposed" rabbits. *U value of Mann-Whitney Utest at 0.05 significance level = 17. Antigen-whole bovine serum.

IMMUNE RESPONSE IN COLD EXPOSURE

visible precipitation in double diffusion tests is dependent upon three factors: (1) the diffusion co-efficient of each of the reactants, (2) their absolute concentrations, and (3) the speed with which the antigen-antibody front can form insoluble complexes.

Assuming that each of the rabbit antibody preparations possesses an equal diffusion co-efficient, and using the same antigen preparation for both systems, the time of appearance of each of the precipitating bands on the Ouchterlony gel diffusion plate should depend upon the concentration of the antibody and/or the affinity or avidity of the antibody for its homologous antigen.

In our evaluation of these data, three methods of measurement were used. In order to analyze the data, (1) the total number of precipitating antigen-antibody complexes was measured at each reading; (2) in an attempt to evaluate the results quantitatively, each of the precipitates was visually estimated and ranked with respect to the total amount and homogeneity of precipitate; and (3) the time of appearance of each of the precipitating bands was recorded. Precipitates were graded one through four based upon assigned criteria, with the sharpest and most well-defined bands being assigned the highest number. Quantitative evaluations were based on the number of bands of precipitate times the intensity of each precipitate; for example, a gel diffusion plate having four sharp, heavy bands of precipitate and one faint, poorly-defined precipitate was assigned a total value of 17 ($4 \times 4 + 1 \times 1$). All of the data, both qualitative and quantitative, were subjected to statistical evaluation by the Mann-Whitney U test. The results of one of these studies are given in Tables I and II.

From these tables it may be seen that a statistical evaluation of both the qualitative data based on the number of precipitating bands in the Ouchterlony gel diffusion plates and the quantitative data based on the number of bands times the intensity of the precipitate failed to reach a level of significance in the Mann-Whitney U test. The U value at the 0.05 significance level is equal to 17. In study groups in which the larger of two independent samples is smaller than 9, tables for U values are not available and the P value is calculated directly (Table III). It may be noted in Table III that differences which are significant at the 0.05 level are obtained in sample 2

NORTHEY

Sample	N	R-Cold	R-Normal	U	P
1	3	0	0		
2	3	6	4	0	.050
3	3	12	11	2	.200
4	3	11	12	4	.500
5	3	15	12	1	.100
6	3	20	18	1	.100
7	3	15	16	4	.500

Table III. Qualitative evaluation of precipitates in gel diffusion of sera from "normal" and "cold exposed" rabbits. Antigen-egg albumin (Ea).

of the egg albumin anti-egg albumin system. However, a significant level was not attained at any other sample in this experiment. An occasional significant value was also found in other experiments, but these findings were not consistent with most of the data. To date, over 500 serum samples have been evaluated in this manner. The same method has also been applied to measurements of the time of precipitation. The results of one series of measurements at closely spaced time intervals is given in Table IV.

"Ring" Precipitin Titrations

The "interfacial technique" has been used extensively as a measure of the antibody "titer" of immune sera. In these studies, serum samples from both study groups were titrated for antibody content using different antigens and varying bleedingschedules. The result of one of these tests using egg albumin as antigen is shown in Table V.

The antibody titrations by the precipitin "ring" technique failed to reveal any consistent trend toward higher antibody levels in either of the study groups. However, when the less complex antigens, egg albumin (Ea) (Table V) and bovine serum albumin (BSA) were used, the antibody levels were somewhat higher in the "cold exposed" group, whereas the animals maintained at room temperatures and

IMMUNE RESPONSE IN COLD EXPOSURE

Qualitative Evaluation

Sample	Time	N	R-Cold	R-Normal	U*
2-1	8 hr.	9	85.500	85.500	40.500
2-2	12 hr.	9	88.500	82.500	37.500
2-3	20 hr.	9	81.000	90.000	36.000
2-4	28 hr.	9	87.500	83.500	38.500
2-5	36 hr.	9	86.500	84.500	39.500

Quantitative Evaluation

Sample	Time	N	R-Cold	R-Normal	U
2-1	8 hr.	9	85.500	85.500	40.500
2-2	12 hr.	9	84.000	87.000	39.000
2-3	20 hr.	9	72.500	98.500	27.500
2-4	28 hr.	9	84.000	87.000	39.000
2-5	36 hr.	9	91.500	79.500	34.500

Table IV. Statistical evaluation of gel diffusion precipitates based on time of precipitation. *U value of Mann-Whitney U test at 0.05 significance level=47. Antigen-whole bovine serum.

Number of Days following Initial Injection	Mean Antibody Titer Cold exposed	Mean Antibody Titer* Non-Cold exposed
4	1-2000	1-1600
10	1-3000	1-3000
17	1-6000	1-4000
24	1-8000	1-5000
30	1-10,000	1-7000

Table V. Mean precipitation titers of "normal" and "cold exposed" rabbits immunized with egg albumin (Ea). *Mean titer calculated on the basis of antibody titers obtained from precipitin titrations of 10 samples of rabbit antiserum.

NORTHEY

using the human serum antigen attained a slightly higher level of antibody. Of interest too is that in the group in which daily blood samples were taken, the additional "stress" created by cold exposure did not significantly alter a steady rise in the antibody level (Table VI and Table VII).

Number of Days Following Initial Injection		Mean Antibody Titer Cold exposed	Mean Antibody Titer* Non-Cold exposed
3		1-2500	1-3000
6		1-5000	1-8000
9		1-5000	1-10,000
12		1-5000	1-10,000
18		1-2400	1-10,000
24		1-6500	1-10,000
48		1-7000	1-10,000

Table VI. Mean precipitin titers of "normal" and "cold exposed" rabbits immunized with human serum. *Mean titer calculated on the basis of antibody titers obtained from precipitin titrations of 6 samples of rabbit antiserum.

Starch Gel Electrophoresis

The technique of electrophoresis in starch gel offers a method of increased sensitivity over that of paper electrophoresis, and under proper conditions, it is possible to resolve as many as fifteen components in human serum (Moretti et al., 1959). This method was utilized for a comparison of the sera from "normal" and "cold exposed" animals in order to detect the possible presence of any "abnormal proteins" which might be formed as a result of cold exposure. Conversely, the failure of the "cold exposed" animal to form any of the "normal" serum sub-fractions could also be recognized. Simultaneous analysis of sera from both "cold exposed" and "normal" rabbits immunized with antigens of varying complexity provides a convenient method for these evaluations.

Samples were analyzed by starch gel electrophoresis in an appa-

IMMUNE RESPONSE IN COLD EXPOSURE

Number of Days following Initial Injection	Mean Antibody Titer Cold exposed	Mean Antibody Titer* Non Cold exposed
1	-	-
2	-	-
3	-	-
4	-	-
5	1-50	1-20
6 **	1-60	1-20
7	1-60	1-20
8	1-100	1-60
9	1-60	1-60
10	1-100	1-60
11	1-100	1-60
12	1-250	1-60
13 **	1-500	1-300
14	1-300	1-500
15	1-1500	1-1000
16	1-1500	1-1000
17	1-1500	1-1500
18	1-3000	1-3000
19	1-6000	1-4000
20	1-6000	1-4500
21	1-7000	1-5000
22	1-7000	1-6000
23	1-8000	1-7000
24	1-8000	1-7500
25	1-10,000	1-8000

Table VII, Mean precipitin titers of "normal" and "cold exposed" rabbits immunized with bovine serum albumin (BSA). *Mean titer calculated on the basis of antibody titers obtained from precipitin titrations of nine samples of rabbit antiserum. **Dates of immunization.

NORTHEY



Figure 2. Starch gel electrophoresis of sera from "cold exposed" (bottom of picture) and "non-cold exposed" (top of picture) rabbits.

ratus of our own design which utilized a borate buffer having an ionic strength of 0.01 in the gel and an ionic strength of 0.03 in the borate bridge. In all cases, samples were analyzed simultaneously using a plastic trench which accommodated two samples at once. A piece of filter paper was saturated with the serum and placed in a slit in the gel. Samples representing both the "cold stressed" samples and "normal" samples were run side by side at a current of 250 volts 5 ma per sample for a five hour period. At the end of the migration period, the strips were cut and stained with amido (black) Schwartz. Permanent records were maintained by sketching the pattern on graph paper and by photographs. A representative starch gel strip is shown in Figure 2.

Thus far, a total of more than 300 serum samples from "cold exposed" and non-cold exposed animals have been analyzed by starch gel electrophoresis, and a critical evaluation of the data has failed to reveal any striking differences. The presence of any "abnormal proteins" in the sera of cold exposed animals was not detected.

However, a serum fraction, which has tentatively been identified as one corresponding to the B lipoprotein of paper electrophoresis (the alpha-2 lipoprotein of immuno-electrophoresis), has been found to be weak or absent in a number of serum samples from

IMMUNE RESPONSE IN COLD EXPOSURE

"cold exposed" animals when compared to the sera from animals maintained at normal environmental temperatures. This fraction has been reported to contain 55 to 66 per cent of the total conjugated lipid in human serum (Urial and Grabar, 1956). A second observation of interest, although not necessarily significant, is that the serum fractions observed on starch gel and stained by Amido (black) Schwartz stain consistently appeared to be more distinct and well defined in the sera of the "cold exposed" animals.

Immuno-electrophoresis

Because of the high degree of resolution possible by immuno-electrophoresis, this technique has proved to be invaluable in the identification of complex antigenic mixtures from a variety of sources (Growle, 1961). We felt that this method would prove to be useful in determining the sensitivity and/or selectivity of the immune mechanism because the sera of animals immunized during periods of cold exposure could be compared with those of animals maintained at "normal" temperature and immunized in an identical manner. The ability of the antibody forming mechanism to respond to a heterogeneous spectrum of antigens present in varying concentrations should provide a sensitive and reliable index of the qualitative immune response. Sera from "cold exposed" and "normal" rabbits were analyzed simultaneously by immuno-electrophoresis, and the results were compared. Serum samples from each of the groups were allowed to migrate in the gel under the influence of an electric current. Following the migration period, the antigen was applied to a center trough and allowed to diffuse into the gel and react simultaneously with each of the separated antiserum preparations. The technique used is shown in Figure 3.

To date, no significant differences have been observed in the qualitative response of "cold exposed" and "normal" animals as measured by analysis of their sera by immuno-electrophoresis. However, application of this technique has been restricted to only forty serum samples due to insufficient time. This phase of the investigation is currently being intensified, and final judgment concerning the applicability of this technique to these studies must await completion of additional experiments.

NORTHEY

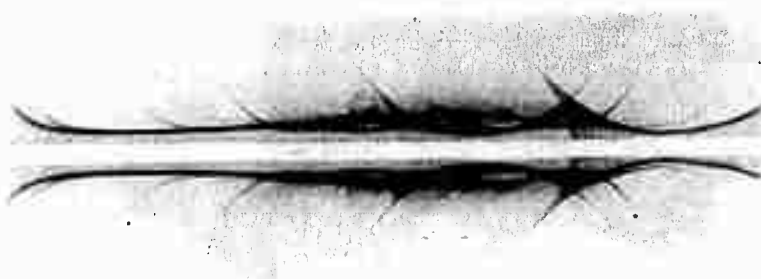


Figure 3. Immuno-electrophoretic analysis of rabbit anti-human serum antibody in "cold exposed" (top of picture) and "non-cold exposed" (bottom of picture) rabbits. 1. Barbitol buffer strength ionic strength 0.375. 2. Stain - thiazine red R.

Quantitative Precipitin Analysis

A limited number of serum samples have been analyzed by the micro-quantitative precipitin method of Lanni (Lanni et al., 1950). The results of this analyses are given in Table VIII.

	N	Antibody Protein mg/ml
Cold Exposed	8	0.428 \pm 0.1548*
Non-Cold Exposed	8	0.3716 \pm 0.0767

Table VIII. Quantitative precipitation analyses of sera from "cold exposed" and "non-cold exposed" rabbits.

It may be noted from the table that the cold exposed rabbits gave slightly higher levels than the non-cold exposed group. This finding is in accord with that of Trapani and Campbell who noted somewhat higher levels in the plasma proteins of cold exposed (1° C to -15° C) rabbits (Sutherland et al., 1958).

IMMUNE RESPONSE IN COLD EXPOSURE

We are currently intensifying this phase of the investigation in an attempt to obtain a much larger sample in order to reach a definitive conclusion. More animals are necessary to lower the standard error to an acceptable level, although population variation here is small, and consequently, this initial estimate of serum protein levels is statistically quite accurate.

Paper Electrophoresis

To date, a total of more than 350 serum samples have been analyzed by paper electrophoresis. In addition, approximately 75 serum samples have been analyzed for glyco-protein content. The results of these evaluations are not available at this time, and are currently being statistically analyzed using the Fisher "t" test by the General Electric computer center at Arizona State University.

DISCUSSION

An evaluation of the data presented here concerning the effect of "cold exposure" on the immune response of rabbits indicates that any "differences" attributable to "cold" per se from both the qualitative and quantitative aspects of immunity are of insufficient magnitude to draw any definitive conclusions.

A fundamental consideration in any investigation of this type concerns what may be construed as "stress" conditions. Again citing the work of Sutherland, Trapani, and Campbell (1958) the rectal temperature of rabbits maintained at 4° C was not significantly different from that of animals maintained at a room temperature of 18° C. In the studies reported here, however, a temperature of 4° C was considered to be a condition of "stress" for rabbits shaved of the bulk of their pelage. This assumption was substantiated by the fact that the animals tended to huddle, their skin was cold to the touch, some shivering was observed, and a sig-

NORTHEY

nificantly large number of animals succumbed from non-specific causes during the course of the various studies in the cold exposed group compared to deaths in those maintained at room temperature.

The quantitative evaluation of sera from each of the groups has provided some interesting information regarding the immune response under conditions of cold exposure. Antigens of varying complexity were used, and the response of the animals measured. The comparatively less complex antigens, egg albumin (Ea) and bovine serum albumin (BSA), provide a sharp contrast to the very complex human serum and bovine serum in terms of multiplicity of antigens to which the antibody forming cells must respond. The failure to find a significant qualitative difference in the experimental and "control" groups indicates that cold exposure does not appreciably affect the total response of an animal to multiple antigen systems. The level of the response, as well as the time required for antibody response, did not differ significantly in any of the gel diffusion studies. In spite of the failure to attain statistical significance in these studies, it must be remembered that these data do not provide a final critical measure of antibody response during hypothermia. Campbell et al. (1951) and Sutherland et al. (1958) have measured the rates of antibody decay during both active immunization and passively administered antibody. These authors report an increased rate in decay of both actively formed and passively administered antibody in cold exposed rabbits. Extrapolation of these findings to the data presented here and increased rate of "decay" may provide a more sensitive measure of the actual difference, and result in the weighing in favor of an increase in antibody production in the "cold exposed" animals. Such an extrapolation is, of course, impossible on the basis of our present knowledge of the mechanisms of protein metabolism and protein turnover in the immunized animal.

It should be pointed out that I am well aware of the inherent dangers in lending too much credence to the results of an evaluation by gel diffusion. Numerous investigators have demonstrated that this procedure is dependent upon a variety of physico-chemical factors, and that this technique, regardless of how critically applied, is subject to error (Glenn, 1959; Jennings et al., 1962).

IMMUNE RESPONSE IN COLD EXPOSURE

Antibody titrations by the "ring" precipitin method are at best only a crude approximation of the antibody concentration in a serum sample. Because dilutions of antigen rather than antibody are used as a measure of "antibody level", the validity of such titrations has been widely criticized (Roffel, 1961). In spite of this fact, this technique has received wide application, and in this investigation, these titrations have provided a simple and convenient method for approximating antibody levels at various dates during the bleeding schedule, and will be supplemented by the vastly more sensitive microquantitative precipitin analyses when time permits.

Baker and Sellers (1960) have observed in "cold exposed" rats the presence of a plasma protein component which they report has a similar electrophoretic mobility to the "transferrin" component of human serum. This component, also known as siderophilin, serves to bind iron in the circulating blood and migrates as a B globulin.

While we did not find the presence of a similar "abnormal" protein in the sera of "cold exposed" rabbits, this does not preclude the possibility of the existence of such proteins. Through the use of different stains such as those for haptoglobins or another immunochemical technique, like the more definitive disc electrophoresis, similar abnormal proteins may be recognized.

The "loss" of the B lipoprotein fraction from the sera of a number of "cold exposed" animals as measured by starch gel electrophoresis cannot readily be explained, and may be due merely to an alteration in the rate of migration. But this observation provides a fertile field for speculation. Masoro (1960) has reported that the cold acclimated rat has an increased capacity to oxidize long-chain fatty acids. A similar increased capacity on the part of the cold exposed rabbit would partially account for a lower level of slow migrating B lipoprotein in this animal. Bertke (unpublished data) and others have reported a decrease in lipid content of the adrenals during stress. Such a decrease could presumably be reflected in a decreased level of circulating lipoprotein. A final speculation concerning a decreased B lipoprotein content of cold exposed rabbits concerns the role of heparin during periods of stress and/or shock. This compound, because of its strong polar properties, is capable of liberating the combined lipid from lipo-

NORTHEY

protein complexes. An increase in heparin would presumably cause a corresponding decrease in the level of circulating lipoprotein. An increase in heparin could presumably also play a role in the increased clotting time in cold exposed rabbits reported by Campbell and Sutherland (1951). It must be pointed out, however, that at the present time, we have no evidence for an increase in heparin in the circulating blood during periods of "cold stress".

While the consistent "tendency" of the sera of "cold exposed" rabbits to form sharper and clearer zones on starch gel provides a poor indication of any "differences" that might exist, this might be due to a greater degree of homogeneity of each of the different protein components measured by electrophoresis, which would result in a more nearly equal rate of migration. This homogeneity would then be reflected as less "trailing" in the stained starch gel pattern.

The value of immuno-electrophoretic analyses of sera from cold exposed and normal rabbits to the present investigation must await further application of this technique. The limitations when this technique is applied must be recognized. Further experimentation may also be necessary to determine optimal buffer pH values, ionic strength, and so forth, all of which are critical in this procedure. In spite of the apparent limitations of this procedure, however, it offers one of the most sensitive measures of qualitative antibody response that is presently available.

Future studies which are planned will include analysis of sera from rabbits exposed to more severe environmental conditions (-15°C), and determinations of possible alterations in lipoproteins, haptoglobins, and other serum sub-fractions. Quantitative measurements of the 7 S and 19 S macroglobulins will be made by density gradient zone ultracentrifugation and chromatography on cellulose ion-exchange columns. It is anticipated that these and future investigations will provide information useful in determining the role of antibody in host-parasite interactions under conditions of hypothermia.

IMMUNE RESPONSE IN COLD EXPOSURE

SUMMARY

Immuno-chemical studies were conducted on sera from rabbits to investigate the qualitative and quantitative aspects of the immune response under conditions of "hypothermia". Rabbits have been subjected to lowered environmental temperature (4°C), shaved of their pelage, and immunized with various protein antigens. Serum samples obtained from varying periods during each of the studies via cardiac puncture have been qualitatively assayed for antibody response by Ouchterlony gel diffusion, immuno-electrophoresis, starch gel electrophoresis, and paper electrophoresis. Quantitative analyses have included "ring" precipitin titrations, quantitative gel diffusion analysis, and micro-quantitative precipitin analysis. In all of the studies the response of the "cold exposed" animals have been compared with the "non-cold exposed" animals treated in an identical manner, except for maintenance at the lower environmental temperature.

LITERATURE CITED

1. Aladjem, F., R. W. Jaross, R. L. Paldino, and J. A. Lackner. 1959. The antigen-antibody reaction. III. Theoretical considerations concerning the formation, location, and curvature of the antigen-antibody precipitation zone in agar diffusion plates, and a method for the determination of diffusion coefficients of antigens and antibodies. *J. Immunol.* 83: 221-231.
2. Baker, D. G., and E. A. Sellers. 1960. Unpublished. Intermediary metabolism: Discussion. Symposium on cold acclimation. *Federation Proc.* 19: Supplement No. 5, p. 133.
3. Bertke, Eldridge. (Unpublished data) Arizona State University.

NORTHEY

4. Campbell, Dan H. 1951. Immunochemical studies of arctic animals. A research report covering the period 1948-1950 for the Office of Naval Research.
5. Crowle, Alfred J. 1961. Immunodiffusion. Academic Press, New York.
6. Freund, Jules. 1947. Some aspects of active immunization. *Ann. Rev. Microbiol.* 1: 291.
7. Glenn, William G. 1959. Some considerations in agar column diffusion analyses. *J. Immunol.* 82: 120-124.
8. Jennings, Robert K., and Morris A. Kaplan. 1962. Implications of qualitative comparative serology. *Ann. Allergy* 20: 15-28.
9. Jungeblut, C. W., M. Sanders, and R. R. Feiner. 1942. *J. Exp. Med.* 75: 611-629.
10. Lanni, F., M. L. Dillon, and J. W. Beard. 1950. Determinations of small quantities of nitrogen in serological precipitates and other biological materials. *Proc. Soc. Exp. Biol. Med.* 74: 4.
11. Moragues, V., and H. Pinkerton. 1944. *J. Exp. Med.* 79: 41-43.
12. Masoro, E. J. 1960. Alterations in hepatic lipid metabolism induced by acclimation to low environmental temperatures. *Federation Proc.* 19: Supplement 5, 115-119.
13. Moretti, J., G. Bouscier, M. Hugou, and L. Hartmann. 1959. *Bull. Soc. Chim. Biol.* 41: 79-87.
14. Ouchterlony, O. 1949. Antigen-antibody reactions in gels. *Acta Path. Microbiol. Scand.* 26: 507-515.
15. Raffel, Sidney. 1961. Immunity. Appleton-Century Crafts, Inc. New York, 1953, p. 155.

IMMUNE RESPONSE IN COLD EXPOSURE

16. Sutherland, G. Bonar, and Dan H. Campbell. 1956. Cold adapted animals. I. Changes in blood clotting and electrophoretic properties of rabbit plasma. *Proc. Soc. Exp. Biol. Med.* 91: 64-67.
17. Sutherland, G. Bonar, Ignatius L. Trapani, and Dan H. Campbell. 1958. Cold adapted animals. II. Changes in the circulating plasma proteins and formed elements of rabbit blood under various degrees of cold stress. *J. App. Physiol.* 12: 367-372.
18. Trapani, Ignatius L., and Dan H. Campbell. 1959. Passive antibody decay in rabbits under cold or altitude stress. *J. App. Physiol.* 14: 424-426.
19. Trapani, Ignatius L. 1960. Cold exposure and the immune response. Symposium on cold acclimation. *Federation Proc.* 19: Supplement No. 5, 109-114.
20. Uriel, J., and P. Grabar. 1956. *Bull. Soc. Chim. Biol.* 38: 1253-1269.

DISCUSSION

BLAIR: Is cold responsible for the changes that have been observed both with regard to the antibody turnover rates, and also with regard to the immuno-electrophoretic studies, or is cold simply a stimulus? In other words, could not the same situation be produced in the laboratory with stimuli other than temperature stimuli, but resulting in experimental preparations metabolically, at least, similar to that induced by cold? In other words, is not cold just a non-specific stimulus, or is cold really responsible for these changes?

TRAPANI: Are you asking about the effect of temperature at the cellular level?

NORTHEY

CAMPBELL: He is talking about hypothermia versus stress. You can have stress without hypothermia. You need to really lower the body temperature, which we haven't done. It has been done in the case of the hibernating squirrels. I think you would have to use an animal of this sort. Of course, this is an adaptation, and you have two problems. It seems to me one is a stress problem and the other is not.

BLAIR: That is right. What I am trying to clarify in my own mind is that the cold exposure is simply one of numerous stimuli that could be used to produce the same situation.

TRAPANI: I think this is true. What we are really dealing with in an animal that is cold exposed are the secondary factors which affect its response.

BLAIR: Could we call this the affect of cold on immune responses, really?

TRAPANI: If you put it in a cold box or out in the snow, a cold stress is imposed, and the index used for measuring the response is attributable to that stress, either directly or indirectly.

BLAIR: Suppose you produce a stress situation through another stimulus?

PREVITE: These responses to various types of stimuli, for example cold, heat, or sound, are not always the same.

MITCHELL: I wonder if we have a veterinarian around here who can tell us whether this is a stress or not. I want to know whether we are really and truly talking about a stress to these animals, or whether we are trying to talk about what would be a stress to a man if we did it to him?

REINHARD: Well, I think if the stress investigators conducted baseline experiments to determine the characteristics of the animal, that all animals would have similarities in their responses to stress. There is only one area in which man is

IMMUNE RESPONSE IN COLD EXPOSURE

different, and that is in the neurological and psychological response. When you talk about man, you have to introduce those factors, also.

BERRY: You are not excluding the psychological factor in animals, are you?

REINHARD: No, but they are quantitatively, and sometimes qualitatively different.

VIERECK: I completely agree with these comments by Dr. Reinhard. One type of baseline experiment would be to expose the animal to the cold temperature and measure food intake over a period of days. I think it is accepted that if the animals consume considerably more food, their metabolism has been higher and they have been under a sort of stress. Then this brings to mind another question. Some animals have been treated in this way, and they do indeed eat more food at certain low temperatures; thus they are eating more protein, and if an animal is consuming more protein for a period of time, will this, all by itself, affect the serum protein electrophoresis patterns regardless of cold exposure? In other words, could cold act via food intake? The protein turnover might be higher whether the stress were cold or whether it were something else.

SULKIN: This is precisely why I asked Dr. Campbell earlier whether he did the experiments with the arctic ground squirrels just at the time that they were going into hibernation. A bat just going into hibernation is not a stressed animal. However, if you are dealing with the same bat species in the cold room in the summertime, he is then in a state of hypothermia, and is in a stressed condition. While I don't intend to talk about electrophoretic changes, we have data showing that there is a difference in the analysis made in hypothermic bats and in hibernating bats, so it is important to emphasize the animal host and the season of the year.

TRAPANI: Is it a qualitative or quantitative difference? Do you pick up different components or the same components in different amounts?

NORTHEY

SULKIN: I am not sure.

CAMPBELL: I suspect you might get a difference in the ratio or distribution in some proteins that might be there in minor amounts. Most of it is the ratio. Instead of being five per cent or ten per cent of the total proteins, it may go up to twelve or fifteen per cent. Now, under stress conditions, I cannot see that anything can happen within an hour or two or even within a few hours, other than changes in fluid balance perhaps; then you get changes in protein like the hibernating squirrel. It looks like its got a high protein concentration, which it has, but the total amount of protein may be actually the same as a normal animal. I don't know what the blood volume was because I didn't measure that.

PREVITE: I agree with what Dr. Sulkin stated a few minutes ago. There is a tremendous difference between hibernation and induced hypothermia. Dr. Lyman at Harvard has just emphasized this in a review on hibernation.¹ In answer to Dr. Mitchell's question about stress, Dr. Cardy,² of the University of Pennsylvania, noted that whether or not an animal is stressed depends upon the host and the conditions of the experiment.

MITCHELL: And the interpretation of the investigator in whether or not he is going to measure it by lowered temperature or the presence of this or the presence of that.

REINHARD: To reply to Dr. Mitchell, there is one more important factor. I wish more people would do comparative work using various species, because no one animal is exactly relevant to man, and they do differ in basic physiology, especially the gastroenterological portion of it.

McCLAUGHRAY: I think it might be well to keep in mind here the distinction between the physiological function of protein metabolism as it might be reflected by the antigen-antibody

¹ Lyman, C. P. 1961. *Circulation* XXIV.

² Hardy, J. D. 1961. *Physiol. Rev.*

IMMUNE RESPONSE IN COLD EXPOSURE

response, and the function of the antibody as a protein component of the serum. These two things may not be exactly the same, and I think that there has been in the past an assumption that we are measuring these two things simultaneously in some of the studies reported. It occurred to me that it might be possible to distinguish these two separate physiological functions in some way.

NORTHEY: In terms of the comments about protein metabolism, one can't say an animal eats more and therefore produces more antibody. The antigenisticity of the substance under investigation makes a difference in probably a multitude of other factors.

CAMPBELL: That is why I was bringing up this last point; the persistence in antigens.

INFLUENCE OF HYPOTHERMIA ON THE ACTION OF BACTERIAL TOXINS

G. Tunevall and T. Lindner

Central Bacteriology Laboratory
Box 177
Stockholm 1, Sweden

ABSTRACT

In hypothermic mice with a body temperature of 22° C to 23° C, given about one DL_{100} of tetanal toxin, the survival was significantly longer than in normothermic ones given the preparatory (Hibernal-Nembutal) treatment but not chilled (54 versus 29.5 hours at 48 hours of hypothermia). This result must be cautiously evaluated. Though hypothermia was not established until three hours after toxin injection, absorption from the subcutaneous site may be slower in hypothermic animals. Further, narcotic pretreatment drugs, though given also to the controls, may be more slowly eliminated by hypothermic mice, resulting in a milder and protracted course of the toxic manifestations. An attempt was done to find out if hypothermia prolonged the time during which toxin could be neutralized by antitoxin. Antitoxin after 15 minutes resulted in survival of all mice. At a toxin-antitoxin interval of four hours no animal was saved, but survival was prolonged and more so in hypothermic mice than was corresponded by the length of hypothermia (80 versus 43 hours at 4 hours of hypothermia). At an interval of 10 hours, survival was shorter in both groups, and the difference between hypothermic and normothermic mice equalled the duration of hypothermia (50 versus 39 hours at 10 hours of hypothermia). These observations must be corroborated by further experiments before they can be safely evaluated. The effects of staphylococcal toxin are less likely to be attenuated by the premedication pertaining to our procedure for inducing hypothermia. On the contrary, a synergism was observed between the narcotic drugs and this toxin, as amounts less than one conventional DL_{100} were sufficient to kill the mice. Also with this toxin, however, the survival was longer in hypothermic mice (3.5 versus 2 hours).

Induced hypothermia has been employed clinically in a variety of conditions, among others in intoxications. As seems often to be the case in connection with hypothermia, animal experimentation has been scarce and tended to lag behind the applications in human beings.

TUNEVALL AND LINDNER

The effect of tetanus toxin on mice subjected to low environmental temperature was studied by Ipsen (1951). The survival after large toxin doses was prolonged in chilled animals, whereas sublethal doses caused more deaths in chilled mice than in those kept at normal room temperature. Increased susceptibility to endotoxins from Gram-negative bacteria of mice held at 5° C and 15° C, when compared to animals at room temperature was found by Previte and Berry (in press). In hypothermia induced so as to avoid stress reactions, no effect on the hematologic or histologic manifestations of staphylococcal exotoxin in rabbits was found by Cole (1960).

EXPERIMENTAL

Material and Methods

Albino mice weighing 20 to 40 gm were numbered serially. The allotment of animals to different experimental groups was done by a random method according to the tables of Fisher and Yates (1953).

For inducing hypothermia the following procedure was used: A subcutaneous injection of 32 mcg/g body weight of Chlorpromazine-HCl is followed after 30 min. by an intraperitoneal injection of half this amount of ethyl-(1-methyl-butyl)-malonyl-carbamide-Na. The mice are then, in their narcotized state, fixed onto suitably formed lead plates with adhesive tape and immersed in a supine position into a 21° C water bath with only the head and part of the thorax above the water. The rectal temperature will be stabilized within one hour between 21.5° C and 23.5° C, and the temperature in lower esophagus will be stabilized about 0.5° C higher. Oxygen and carbogen are administered continuously to the chamber formed by the water bath and its fairly tightly closed cover.

Rewarming starts with slowly raising water temperature to 33° C over a 4 to 5 hour period. When they begin to show activity, the mice are freed from the lead plates, dried, transferred to their

HYPOTHERMIA AND BACTERIAL TOXINS

original containers, and placed into an air incubator generally at 35° C for the first 2 hours, then at 31° C for 24 hours. Humidity is kept around 50 per cent, and oxygen administration is continued. After this period, the mice can be kept under normal conditions.

Normothermic controls are given the same premedication, whereby rectal temperature decreases by 2° C to 5° C, but are immediately transferred to the air incubator, and from that point treated as above.

Tetanus and staphylococcal toxins and antitoxins were obtained from the Swedish State Bacteriological Laboratory. In preliminary experiments on normal mice of our breed, their MLD₁₀₀ neutralizing doses were determined. The routes of administration of these products will be given for each experiment.

RESULTS

In a first experiment presented in Fig. 1, twenty mice were given one MLD₁₀₀ of tetanus toxin subcutaneously. Two hours later hypothermia was induced in ten mice which were then kept in this state for about 48 hours. The other ten mice received pretreatment only. Tetanic manifestations were only slight in the hypothermic animals in comparison with those of the controls, but at rewarming, after 48 hours, paroxysms grew more frequent and intense. One normothermic mouse was lost because it was severely bitten, and two hypothermic ones were lost from drowning. Therefore, recording of survival times could be done only in nine normothermic mice and in eight hypothermic mice, as reported in Fig. 1.

The average survival within the hypothermic group was significantly longer than among normothermic controls, as is visible from Fig. 2. In addition, it can be mentioned that two mice only died during the period of hypothermia, two during the first stage of rewarming in the water bath, one during the stay in air incubator at 35° C, and the remaining three 5 or 6 hours after the change of

TUNEVALL AND LINDNER

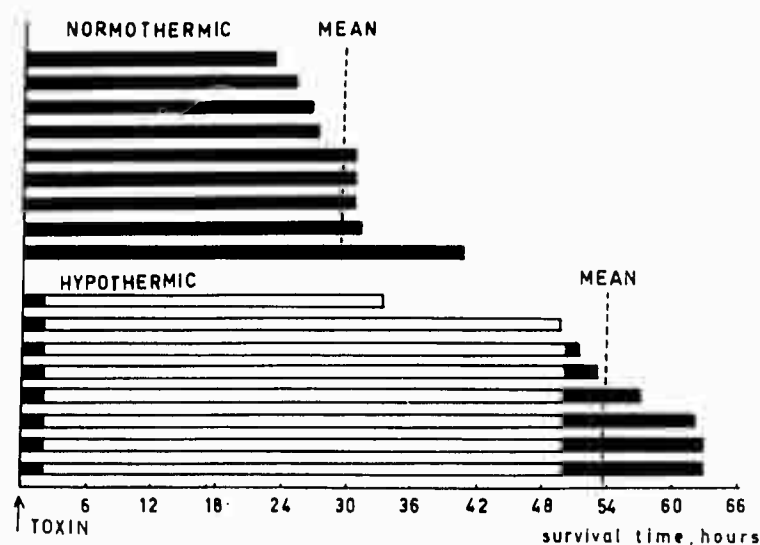


Figure 1. Survival times in mice given one MLD₁₀₀ of tetanus toxin. White parts of columns mark period of hypothermia. Number of mice: Normothermic 9, hypothermic 8.

Group	Number of mice	Survival in hours	Diff.	t	P
Normothermic	9	29.5 ± 1.7	24.5	6.2	< 0.001
Hypothermic	8	54.0 ± 3.5			

Formulae used: $s = \pm \sqrt{\frac{\sum(x-\bar{x})^2 + \sum(y-\bar{y})^2}{n_x + n_y - 2}}$, and

$$t = \frac{D}{s} \sqrt{\frac{xy}{x+y}}$$

Figure 2. Survival times in hours ($M \pm e(M)$) of mice after subcutaneous injection of equal doses of tetanus toxin. Start of hypothermia 2 hours, rewarming 48 hours after the injection.

HYPOTHERMIA AND BACTERIAL TOXINS

temperature to 31° C.

The above results could at least partially be due to a slower fixation of the toxin to susceptible cells in hypothermic animals. Therefore, new experiments were set up in order to find out if hypothermia prolonged the time during which the toxin remained free to be neutralized by antitoxin.

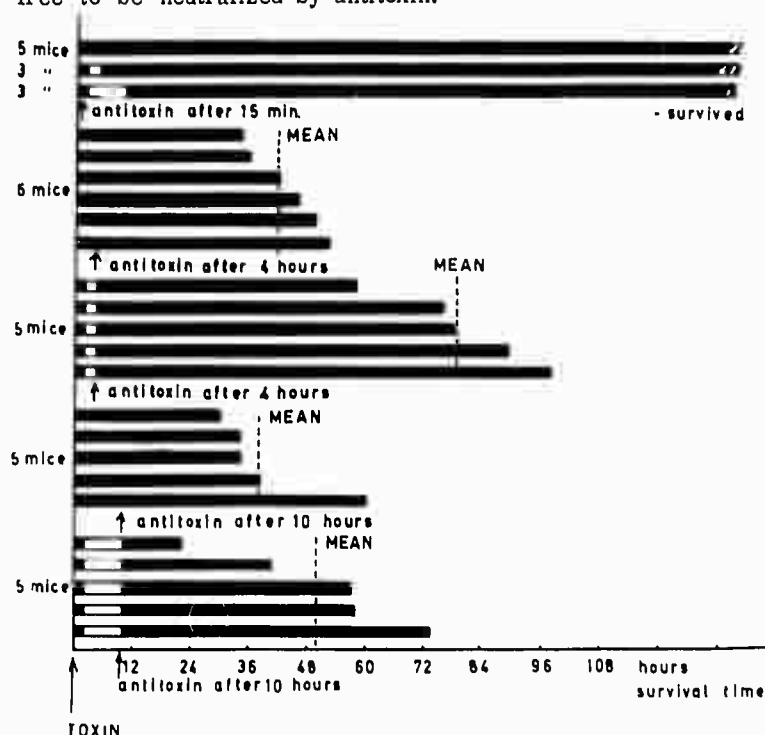


Figure 3. Survival times in mice given one MLD₁₀₀ of tetanus toxin and, after intervals of 15 minutes, 4 hours and 10 hours, one neutralizing dose of antitoxin. White parts of columns mark period of hypothermia, induced two hours after the toxin injection and maintained for two or eight hours.

The results of one experiment are reported in Fig. 3. In all, 35 mice were given one MLD₁₀₀ subcutaneously. They were allotted to three groups containing 11, 12, and 12 animals respectively. In the first group, one neutralizing dose of antitoxin was given, also subcutaneously, 15 minutes after the toxin injection. About 1-1/2

TUNEVALL AND LINDNER

hours later, hypothermia was induced in six mice and maintained for 2 hours in three animals and for 8 hours in the other three. Five mice were kept normothermic. In this group all eleven mice survived, but it is not included in the table.

In the second group six mice were made hypothermic 1-1/2 to 2 hours after the toxin injection, and the other six kept normothermic. All animals got antitoxin 4 hours after the toxin, and then the re-warming of the hypothermic animals was immediately begun. In this group no animal was saved, as seen from Figure 3, but the survival was prolonged in comparison with the first experiment; especially so in hypothermic animals. The difference effected by the hypothermia was much larger than corresponded to the length of the hypothermic period itself (80 versus 43 hours at two hours of hypothermia).

The third group received antitoxin 10 hours after the toxin, and the re-warming of hypothermic mice was also begun after 10 hours. In other respects this group was treated as the second. The survival was still prolonged, but the difference between hypothermic mice and controls was only equal to the length of hypothermia (50 versus 39 hours at 8 hours of hypothermia). This experiment is also presented in Fig. 4, which states the significance of the observations.

As will be discussed later on, the effect of tetanus toxin may be influenced by irrelevant factors. The next part of the study was therefore performed with staphylococcal toxin which was, as well as antitoxin, always administered intravenously in order to avoid differences in absorption from local sites of injection. Preliminary experiments had shown that a dose of staphylococcal toxin large enough to kill all mice in the groups resulted in very short survival, generally ranging between 1 and 4 hours. It was further found that about 0.8 MLD₁₀₀ was enough to kill mice which had received the pretreatment whether they were made hypothermic or not.

In the first series of experiments, the influence of hypothermia on the survival times after lethal doses of the toxin was investigated. Groups of normothermic mice which received the

HYPOTHERMIA AND BACTERIAL TOXINS

Number of group	1.	2.	3.	4.
Toxin-antitoxin interval, hours	4		10	
State	Normo-thermic	Hypo-thermic	Normo-thermic	Hypo-thermic
Number of mice	6	5	5	5
Survival time in hours	52 45 42 34 49 36	98 77 58 76 89	60 34 38 31 34	58 57 41 74 22
$M \pm e(M)$	43 ± 2.9	80 ± 6.8	39 ± 5.3	50 ± 8.8

Comparison 1.-2.: $D = 37$. $t = 4.8$ $P < 0.001$
2.-4.: $D = 30$. $t = 2.7$ $0.05 > P > 0.02$

Figure 4. Survival times in hours of mice given one neutralizing dose of antitoxin serum 4 versus 10 hours after the tetanus toxin injection. Start of hypothermia 1-2 hours after the toxin injection, warming up immediately after the injection of antitoxin. (5 normothermic mice, 3 hypothermic for 3 hours, and 3 hypothermic for 9 hours, all receiving antitoxin after 15 min., survived).

pretreatment only were run. The toxin injection was always made after the start of hypothermia. The experiments differed slightly as to the period of hypothermia preceding the toxin injection and the dose of toxin, but hypothermia was always maintained to the end of the experiments. The results are given in Fig. 5.

Thus, in all groups, the average survival was longer in hypothermic mice. The differences within the groups were significant, and if all the material is taken together, they were even more significant. It was next attempted to study the influence of hypothermia on the time during which staphylococcal toxin could be neutralized by antitoxin to a degree sufficient to save the mice or at least prolong their survival in a manner similar to

HYPOTHERMIA AND BACTERIAL TOXINS

Number of group	1.	2.	3.	4.
Toxin-antitoxin interval, hours	4		10	
State	Normo-thermic	Hypo-thermic	Normo-thermic	Hypo-thermic
Number of mice	6	5	5	5
Survival time in hours	52 45 42 34 49 36	98 77 58 76 89	60 34 38 31 34	58 57 41 74 22
$M \pm e(M)$	43 ± 2.9	80 ± 6.8	39 ± 5.3	50 ± 8.8

Comparison 1.-2.: $D = 37$, $t = 4.8$ $P < 0.001$
2.-4.: $D = 30$, $t = 2.7$ $0.05 > P > 0.02$

Figure 4. Survival times in hours of mice given one neutralizing dose of antitoxin serum 4 versus 10 hours after the tetanus toxin injection. Start of hypothermia 1-2 hours after the toxin injection, warming up immediately after the injection of antitoxin. (5 normothermic mice, 3 hypothermic for 3 hours, and 3 hypothermic for 9 hours, all receiving antitoxin after 15 min., survived).

pretreatment only were run. The toxin injection was always made after the start of hypothermia. The experiments differed slightly as to the period of hypothermia preceding the toxin injection and the dose of toxin, but hypothermia was always maintained to the end of the experiments. The results are given in Fig. 5.

Thus, in all groups, the average survival was longer in hypothermic mice. The differences within the groups were significant, and if all the material is taken together, they were even more significant. It was next attempted to study the influence of hypothermia on the time during which staphylococcal toxin could be neutralized by antitoxin to a degree sufficient to save the mice or at least prolong their survival in a manner similar to

TUNEVALL AND LINDNER

Group	1.		2.		3.		4.	
State	N	H	N	H	N	H	N	H
Survival, minutes	105	225	130	180	80	200	110	345
	60	160	160	150	190	190	110	370
	100	250	120	215	100	240	140	100
	30	150		210	150	230	50	195
	100	150		210	120	180	70	380
	100	195		220				
	75	240		230				
	90	230		265				
	80	200		230				
	80	230						
M	82	203	137	212	128	208	96	278
e(M)	± 7.3	± 12.0	± 12.0	± 10.8	± 19.3	± 11.6	± 16.0	± 55.7
Diff.	121		75		80		182	
t-value	8.58		3.64		3.55		3.14	
Degrees of freedom	18		10		8		8	
P	< 0.001		0.01-0.001		0.01-0.001		0.02-0.01	

Total N: 102 \pm 7.6 Diff. 118. $t = 8.09$. dF 50. $P < 0.001$
Total H: 220 \pm 11.5

Figure 5. Survival times in normothermic versus hypothermic mice given one MLD₁₀₀ of staphylococcal toxin. Hypothermia induced about 5 hours before the toxin injection. (N = normothermic. H = hypothermic).

the work on tetanus toxin. In order to establish suitable experimental conditions, a number of neutralization tests were first set up in normothermic mice given only the pretreatment. The results are reported in Fig. 6 and Fig. 7.

As the number of mice was small and as the results emanate from several experiments, no statistical treatment has been done, but generally, there was a good correlation between the length of the toxin-antitoxin interval and the survival times. Essentially, the experiments differed in one respect only; in one group, the antitoxin dose was increased from one to four neutralizing units, and the results of this experiment are reported in Fig. 6 and Fig. 7.

HYPOTHERMIA AND BACTERIAL TOXINS

Survival minutes	< 60	61- 75	76- 90	91- 120	121- 150	151- 180	181- 240	241- 480	00
Interval minutes									
1									5/7
2				4	1			2	/5
3-5	1			1	1	2	1	1	/1
6-15	2		1	3	1	1	1		
16-25	1	2			/1	1/3			
26-45					/1				
46-60			/1						

Figure 6. Survival times of 51 pretreated but normothermic mice after intravenous injection of one lethal dose of staphylococcal toxin, followed by one or (bottom right in the columns) four neutralizing units of antitoxin after different intervals.

Common to all groups was the observation that increasing toxin-antitoxin interval diminished the ability of antitoxin to prolong the survival. The larger dose of antitoxin gave more absolute survivals and at longer toxin-antitoxin intervals increased survival times.

As to the influence of hypothermia on the time relationships described in the Figures 6 and 7, the work in this area is in its beginning stages. Only one of the experiments has contained five animals made hypothermic about five hours prior to the injection of toxin. They all represent a toxin-antitoxin interval of two minutes. The average survival time of these mice was 284 minutes, and the result is marked as a single point in the figure. This is situated well above the curve for normothermic mice given the same antitoxin amount, one neutralizing dose, but there is no statistical difference between this average and that of the normothermic mice in the same experiment ($t = 1.7$; $dF 8$; $P > 0.1$).

TUNEVALL AND LINDNER

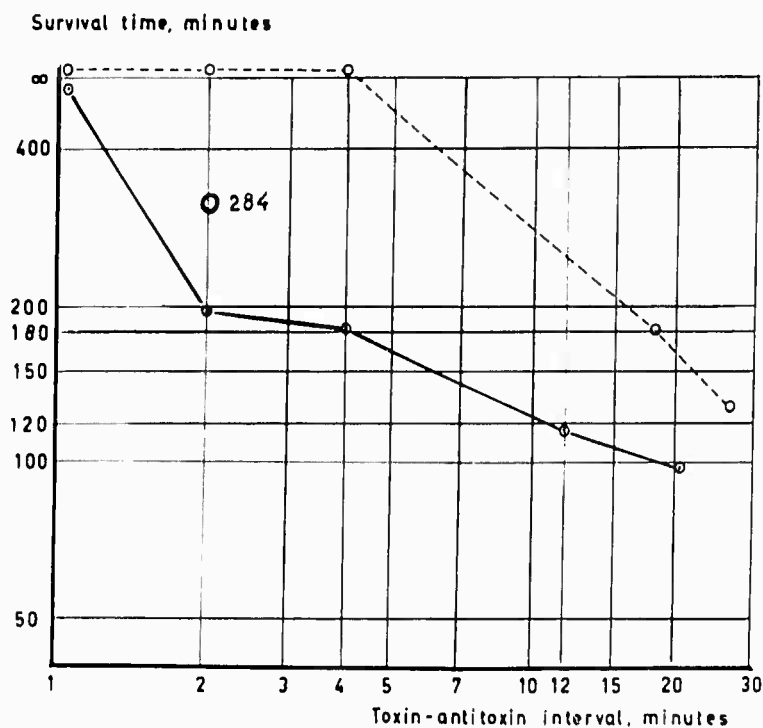


Figure 7. Average survival times of groups of normothermic mice given one MLD_{100} of staphylococcal toxin and, after different intervals, one or four (broken line) neutralizing doses of homologous antitoxin. Same average for a group of five hypothermic mice is marked as a single point.

DISCUSSION

It should first be pointed out that our experiments involve a procedure for attaining hypothermia by the elimination of the normal thermoregulation; thus, hypothermia is reached without stress reactions.

HYPOTHERMIA AND BACTERIAL TOXINS

The first experiment indicated a significantly prolonged survival time and milder tetanic manifestations in hypothermic mice. These mice, however, got more pronounced tetanus and died at or very soon after rewarming. To this result, which may mean simply that a postponement of the toxic effect has come to an end, may be contributed the trauma induced by the rewarming process itself. The possibility also remains that warming up effects a compensatory overnormal metabolism, thus giving the toxin an especially good access to susceptible cells.

If the protective effect of hypothermia is due to a retarded fixation of the toxin to its receptor cells, hypothermia should prolong the period during which the toxin may be neutralized by antitoxin before its entry into these cells. Too few experiments have been made to allow a safe verification of this assumption, but it is interesting to note that with a toxin-antitoxin interval of four hours, a two hour period of hypothermia increased the average survival to not less than 37 hours. Further experiments are planned to investigate whether this increase may be converted to a lasting survival if an adequate toxin-antitoxin interval is chosen.

The evaluation of these results with tetanus toxin must be made with great caution. The injection of toxin was made subcutaneously and though hypothermia did not start until about 2 hours after the injection, part of the toxin may have been more slowly absorbed in hypothermic animals. Furthermore, narcotics, as a rule, have an attenuating effect on tetanic manifestations. There are good reasons to believe that the drugs given as pretreatment are more slowly eliminated in hypothermic animals, and this may account for at least part of the protective effect of hypothermia. Therefore, staphylococcal toxin was chosen for the subsequent tests. In this case the situation is apparently reversed, in that the effect of this toxin seems to be enhanced by the narcotics used for pretreatment.

Hypothermia was also found to prolong the survival time after the injection of staphylococcal toxin. On the basis of the same considerations as for tetanus toxin, a series of neutralization tests have been begun which have already indicated a fairly good correlation between the time allowed to pass between the injections of toxin and antitoxin, and the ability of antitoxin to prolong the survival time in

TUNEVALL AND LINDNER

normothermic mice. The ability of hypothermia to alter this relation is not yet verified, but according to a first experiment, it seems probable that it will be.

SUMMARY

In hypothermic mice with a body temperature of 22° C to 23° C given lethal doses of tetanus toxin, the survival time was significantly longer than in normothermic controls given a preparatory (Hibernal-Nembutal) treatment but not chilled. The tetanus was also less pronounced in hypothermia, but increased at the rewarming procedure. During or soon after rewarming, the animals died.

The ability of antitoxin to prolong the survival in normothermic mice varied with the interval between the injection of toxin and antitoxin administration. When hypothermia was maintained during part of this interval, the survival was significantly more prolonged, and much more than corresponded to the length of the hypothermic period.

For several reasons, these results must be cautiously evaluated, but they suggest that the fixation of toxin to susceptible cells is retarded in hypothermia.

In another series of experiments with staphylococcal toxin, similar results were obtained. Hypothermia prolonged survival and the neutralizing effect of antitoxin diminished when the toxin-antitoxin interval increased. That hypothermia may prolong the period during which neutralization is possible is not yet known, but a first experiment of a series to be continued in the future points in this direction.

HYPOTHERMIA AND BACTERIAL TOXINS

LITERATURE CITED

1. Cole, W. R. 1960. Studies in hypothermia and staphylococcus toxin shock. Dissertation Abstr. 20: 4373.
2. Fisher, Ronald A., and Frank Yates. 1953. Statistical tables for biological, agricultural, and medical research. Hafner Publ. Co., Inc.
3. Ipsen, J. 1951. The effect of environmental temperature on the reaction of mice to tetanus toxin. J. Immunol. 66: 687.
4. Lindner, T., and G. Tunevall. 1958. Hypothermia and infection. I. Influence of hypothermia on antibody formation in mice in the secondary response to typhoid-H-antigen. Scand. J. Clin. Lab. Invest. 10: 142.
5. Previte, J. J., and L. J. Berry. In press. The effect of environmental temperature on the host-parasite relationship in mice. J. Infect. Dis.

DISCUSSION

SULKIN: I think it might be of interest to recall some experiments that were reported in the early twenties by Bronfenbrenner and Weiss¹ in which it was shown that ether anesthesia, alone and in combination with specific antitoxin, decreased mortality in experimental botulism in mice. On the basis of these early studies, I became interested in the effect of anesthesia on experimental viral infections² and found that many animals

¹ J. Exp. Med. 1924, 39: 517-532.

² Sulkin et al. 1946. J. Exp. Med. 84: 277-292.

TUNEVALL AND LINDNER

would survive infection with Western equine virus if they were kept under anesthesia for prolonged periods of time, and furthermore, by using hyper-immune serum additional animals would survive.³

CAMPBELL: What is your prolonged period of time?

SULKIN: Three 4-hour periods of diethyl ether anesthesia were used. A similar underlying mechanism may be involved, since during anesthesia the temperature falls as a result of diminished muscular activity and increased heat loss.

BLAIR: I think this is a very interesting piece of work, and while the experimental model, Dr. Tunevall, is quite different than the one which I will discuss the day after tomorrow, I believe that the philosophies are probably going to be quite similar. There are two probable foci of activity, one which you have emphasized; that dealing with the toxin-antitoxin activity, that is, the activity of the organism depending on the organism itself. And of course, the other site of action is the host itself; that is, the overall physiological integrity of the host as a result of the induced infection. I think that the point that you made is that hypothermia seemed to postpone the activities. I have found this to be very much the same situation, using gram negative coliform bacillus in my own studies. I hate to give away all my thunder, but I think it is apropos since your work does dovetail so well with it. It is simply that the hypothermia doesn't really alter these things to a tremendous extent permanently. It is particularly striking that upon rewarming there was a very high death rate. This re-emphasizes the matter somewhat and the picture of death and prevalent death is identical upon rewarming.

BLAIR: The point is, that we have to consider very carefully the level of hypothermia that we are talking about, and using in the experimental model, particularly with relation to overall physiological changes as presumably benefits the un-

³ Sulkin et al. 1945. *Proc. Soc. Exp. Biol. Med.* 60: 163-165.

HYPOTHERMIA AND BACTERIAL TOXINS

fortunate situation which it creates, and then, of course, the level of hypothermia which may have a more direct effect upon the organisms in question.

PREVITE: I am curious, Dr. Tunevall, as to why you didn't make your normothermic mice swim for two hours in a water bath at thirty-seven degrees just to make the two groups comparable. You tied the mice down and made them swim in cold water, but not warm water.

TUNEVALL: I don't know how to get them to stay in the water bath.

MIRAGLIA: If you place the animals in the containers that have very smooth vertical sides, there is no difficulty in keeping the animal swimming continuously for over an hour.

MONCRIEF: At Denver, they tried that and they just stiffened their tails up and stood on them, or else balanced their chins and their tails on both sides of the container.

BLAIR: Apparently I should harbor resentment, Dr. Tunevall, in your initial observation that the cart came before the horse with regard to using hypothermia. I have been "guilty". I refuse to take the stand or stand court trial on this, but hypothermia probably has been exercised rather liberally with patients before there has been, shall we say, adequate research or experimental investigation. I do wish to state, though, that there has been a tremendous amount of research in hypothermia for many years before this modern, so-called era of clinical application. But it is true, however, that there are many facets that we know very little about, particularly with regard to infections, on the hazards of hypothermia. Part of this has been demonstrated by virtue of the fact that there has been a good deal of difference as reported on results and literature on experimentally induced infections using pneumococcus, for example. The animals were cooled -- I believe these were mice -- to very profound levels of 20° C. While there was one report of some improvement of survival, actually there were other reports which indicated a higher death rate. I think that Dr. Eisman

TUNEVALL AND LINDNER

did this. I was in Colorado last March and talked with one of his young gentlemen. They have since repeated this work using more moderate levels of cooling -- 30° C -- and the opposite result was obtained. There was a much more significant rate of survival at the more moderate level of cooling, and this obviously related to the host problem that I mentioned.

TUNEVALL: My characterization of the situation relates to the early 1950's when hypothermia was already being used therapeutically in several clinical conditions. I don't think they knew very much what they were doing then.

BLAIR: I discussed it a few moments ago. They used artificial hibernation, and I hate those words.

CAMPBELL: Do you think new immunomechanisms, antibodies, play any part in it?

TUNEVALL: In this connection, no, I don't think so.

CAMPBELL: But there aren't any so-called natural antibodies on the antigens that you were testing?

BERRY: In that connection, at the meetings in Montreal it was said by one of the speakers on a symposium⁴ that all animals have a very high immunity, not a natural immunity. It is acquired through contact with staphylococci and this is the reason why any immunization against staphylococci is so unsuccessful. They are already maximumally immunized. This is a concept that never occurred to me, but maybe it has some validity, at least in regard to the staphylococcal antibodies not normally present as a result of the contact.

TUNEVALL: I think that the only way to bring active antibody formation into this picture would be to arrange for a secondary response.

⁴ Dr. David Rogers, Vanderbilt University.

HYPOTHERMIA AND BACTERIAL TOXINS

CAMPBELL: I was thinking of the situation in which the antibodies are already there and they are less operative. I do not know the mechanism on neutralization, but you do have stress conditions in which you can reduce shock, particularly in mice, by injecting cortico steroid. If you inject it ten or fifteen minutes before you inject antigen, you can reduce the hypersensitivity of the reaction.

BERRY: Dr. Previte did some experiments with staphylococci.

PREVITE: Yes, that was part of a study on cold exposure. However, since the experiments were of short duration, and since rectal temperatures of staphylococcus toxin injected animals were not measured, and hypothermia was not induced, the results are probably not applicable to Dr. Tunevall's findings.

BERRY: No, they are not directly so, but there was no effect of cold exposure under your conditions?

PREVITE: No, because the number of animals used was not large enough. However, the results did indicate that a significant effect would have been demonstrable with a larger number of mice.

MITCHELL: Saint Patrick did such a good job over in Ireland, but would the same mechanisms that you are talking about relative to hypothermia, and with your so-called antitoxin, work also for venoms of snakes?

BERRY: You are asking Dr. Tunevall?

MITCHELL: Yes.

ANDREWES: He didn't know about Saint Patrick.

MITCHELL: Saint Patrick did a good job of cleaning up Ireland of snakes for the poor. Actually, what I am talking about is whether or not the mechanism you have described, say, for staph-toxin, and for one of your other toxins, works equally well for venoms of what we call our rattle snake, or in our

TUNEVALL AND LINDNER

country, the coral snake, or perhaps the cobra; now, whether or not these could be employed, because we do have situations in which this occurs, becomes a very important facet of a military operation.

TUNEVALL: I can only guess in that connection, and guesses are not sure.

BERRY: You have not used the intravenous route of administration of toxins and antitoxins?

TUNEVALL: Yes, I have injected by the intravenous route.

PREVITE: There have been some reports in which rabbits rendered hypothermic and infected with staphylococcus manifested prolonged survival compared to homeothermic infected controls. Staphylococci were injected into the bones of the rabbit.⁵

BLAIR: I think an important matter here is that there have been various types of experimental models also in administration as well as type of organism used, but the important thing to me is the fact that while survival was prolonged, all of the animals succumbed. There have been no experiments with permanent survival, and that is a very important matter because, as I will discuss again in detail the day after tomorrow, I think that in the viewpoints concerning the role of "therapeutic" hypothermia, we are going to need some very definite clarification. Obviously, the situation which does not produce full, long-term survivals can hardly be considered efficacious.

PREVITE: Yes, but it might be used as an adjunct with something else.

WALKER: Or it may provide you with time to do something like administer antitoxin.

⁵ Grechishkin, D. K. 1956, Eksperim. Khirurgia.

HYPOTHERMIA AND BACTERIAL TOXINS

BLAIR: Yes.

CAMPBELL: Concerning the basic immune mechanism, this occurred to me. I wonder if anyone has studied the effect of hypothermia on the threshold reaction to histamine acetylcholine, or so-called slow reacting substance. That would be a fairly interesting problem.

NORTHEY: Well, I'd just like to add to that a little. We have done some preliminary studies in which we sensitized cold exposed and control Guinea pigs to egg albumin and later on moved the uterine horn and/or a strip of smooth muscle from the intestine. With both the egg albumin antigen and histamine we stimulated these tissues in the cold exposed and non-cold exposed Guinea pigs. The responses were measured on a physiograph. In these preliminary experiments which were made with ten or twelve animals per study group, we were able to see no significant differences in the responses of the cold exposed animals from those in the controls.

BLAIR: I can't remember the details, but the Schwartzman phenomenon was studied. I don't know if this falls strictly into the category that you mentioned, but the cutaneous manifestations are delayed, and if they do appear, they are considerably less.

TRAPANI: Is that because skin temperature is different from core temperature?

BLAIR: No, this is a stabilized state and during stabilized hypothermia, the gradient between the skin and the core is very much the same in the hypothermia as it is without the hypothermia, so the skin temperature, of course, is lower, and this might be part of that. The blood flow to the skin has been measured in hypothermia and during this stabilized state. It is reduced, but not considerably. It is markedly reduced, of course, during the period of cooling, but in the so-called steady state, the blood flow to the skin is fairly substantial.

TRAPANI: But you can still have an actual temperature effect,

TUNEVALL AND LINDNER

per se, on the cellular mechanism?

BLAIR: Oh, yes. Of course, the skin and temperature, per se, is lower; however, it gradually begins to rise in the room temperature environment.

**INFLUENCE OF COLD
ON HOST-PARASITE INTERACTIONS**

PART II

Editor

ELEANOR G. VIERECK

ARCTIC AEROMEDICAL LABORATORY
FORT WAINWRIGHT
ALASKA

1963

EFFECT OF LOW AMBIENT TEMPERATURES ON SPECIFIC AND NONSPECIFIC RESISTANCE

Fred Miya, Stanley Marcus and LeGrande J. Phelps

University of Utah
College of Medicine
Salt Lake City, Utah

ABSTRACT

Adult albino mice (Mus musculus) have been employed in a series of experiments to determine the effect of acute and chronic low temperature exposure on resistance to bacterial disease. Disease organisms used were Klebsiella pneumoniae and Staphylococcus aureus. Animals challenged with these organisms had been previously immunized with the specific agent or had been pretreated with zymosan or Escherichia coli endotoxin. Groups of mice kept at 21° C were compared to similarly treated groups kept at 2° C for 30-45 days. Also, mice challenged at 21° C were then placed at 2° C. The mice challenged at 2° C were kept at this same temperature, caged either singly or in groups. The results show that under these conditions specific immunization affords significant protection as compared to nonspecific immunization of the animals. The degree of resistance induced by nonspecific immunization is significant by comparison with the control animals. The extent of protection is decreased if the animals are acutely cold stressed as compared to chronic cold stress. Also, specific immunization does not protect animals as well if they are caged individually at the cold temperature as compared to being caged in groups; thus, psychological factors of isolation should not be disregarded. It is concluded that specific immunization affords increased resistance compared to nonspecific immunization; however, the degree is dependent on factors such as grouping and chronicity of exposure.

Reported results vary concerning the effect of exposure of experimental animals to cold on virus-induced diseases. In some cases resistance has been found to decrease, in other cases resistance has been found to remain unchanged or to increase. Different routes of challenge, viral agents, animal species employed, and variations in caging, such as individual or grouped, are among the variables which may account for apparently diverse results reported. Experiments were conducted with one strain of adult albino Mus musculus to determine the effect of acute and chronic exposure of these animals on induced viral disease. The mice were kept at either 2° C or room temperature (ca. 21° C). The viral agent chosen was a strain of Coxsackie B-5 which will infect the mice following intraperitoneal injection. Results indicate that: (1) Acute exposure followed by challenge results in lowered resistance. (2) Specific immunization affords significantly increased protection which is not reduced by acute exposure. (3) Nonspecific immunization enhances resistance above that shown by untreated animals, but the extent of resistance is less than that achieved by specific immunization. If the animals are first acclimatized to cold and then challenged, results are changed in the following manner: (1) Acclimatized mice are capable of withstanding challenge doses that

MIYA, MARCUS AND PHELPS

kill challenged and acutely exposed animals. (2) Specific immunization increases resistance of mice kept at 2° C, and this immunity is of the same magnitude as in animals kept and immunized at room temperature. (3) Nonspecific immunization does not increase resistance of cold acclimatized mice.

Numerous reports on the influence of environmental temperature on host-parasite relationship have appeared in the following literature: Pasteur, Joubert, and Chamberland, 1878; Lillie et al., 1937; Fay and Henny, 1938; Armstrong, 1938 and 1942; Smith and Fay, 1939; Bischoff and Long, 1939; Sarracino and Soule, 1942; Fuller, Brown, and Mills, 1941; Goldfeder, 1941; Wallace, Wallace, and Mills, 1942 and 1944; Mills and Schmidt, 1942; Muschenheim et al., 1943; Sulkin, 1945; Ipsen, 1952; Walker and Boring, 1958; Sulkin et al., 1960; Miya et al., 1962; Miraglia and Berry, 1962; Previte and Berry, 1962. There is general agreement that the physical environment can alter or influence the course of disease; however, isolating the specific factors involved remains difficult. Consequently, the reports of results obtained by workers in this field are frequently conflicting even though the same challenge agents and animal species may have been employed.

During the past two years experiments have been conducted in our laboratory to test the hypothesis that preceding or following exposure to low temperatures, the capacity of experimental animals to resist local or systemic infectious diseases is (a) unchanged or (b) increased or decreased. Presumably, changes observed would be mediated by humoral, cellular, or vaguely defined physiological factors which may be isolated or otherwise identified for study.

Our experiments have proceeded along three lines: (1) investigation of the effect of low ambient temperature on host-parasite relations in both unacclimatized and acclimatized animals following experimental infection; (2) investigation of alteration of specific and nonspecific resistance of animals exposed to cold stress; (3) investigation of the influence of low ambient temperature on the progress of viral neoplastic disease in mice.

SPECIFIC AND NONSPECIFIC RESISTANCE

MATERIALS AND METHODS

In all the experiments adult albino mice (Mus musculus) were randomized as to sex, age, and weight. The average weight of the animals at the initiation of any experiment was 21 gm. The ambient temperatures employed were 21° C and 2° C. The temperatures of the rooms did not vary more than $\pm 1.5^{\circ}$ C. The mice were placed at 2° C following treatment and challenge at 21° C (acute exposure of unacclimatized animals) or exposed to 2° C for varying periods, treated, challenged, and observed at 2° C (acclimatized animals).

Klebsiella pneumoniae was obtained from departmental stock cultures, and Staphylococcus aureus, strain Fritchie, was obtained from Doctor R. D. Higginbotham, University of Texas Medical Branch, Galveston, Texas. The organisms were maintained on blood agar. Single colonies were transferred to tryptose phosphate broth (Difco) or solid media and incubated at 37° C for 18 hours when needed.

Viruses used in these experiments were obtained from departmental stock and from Doctor Duard L. Walker, University of Wisconsin, Madison, Wisconsin. The viruses were propagated and assayed on monkey kidney cells using technic described by others (Dulbecco and Vogt, 1954; Youngner, 1954; Bubel, 1958; Bailey, 1960).

Vaccines were prepared for specific immunization from the bacterial and viral agents by the use of formalin inactivation. The mice were immunized with intraperitoneal injections of the vaccines contained in 0.1 ml volume. Seven days were allowed to lapse before challenge. Zymosan (lot OB 298, Fleischmann Laboratories, Stamford, Connecticut) and Escherichia coli endotoxin (Difco 0293, E. coli 055:B5) were used as nonspecific immunizing agents. The details of the preparation and immunization schedules have been previously described (Miya, Marcus, and Perkins, 1961; Marcus et al., 1961a; Marcus et al., 1962).

Measurements of the mouse core, shell, and upper respiratory cavity temperatures were obtained with calibrated probes (Electric

MIYA, MARCUS AND PHELPS

Universal Thermometer, type TE3, Chemical and Pharmaceutical Industry Company, Inc., New York). The rectal probe was inserted 2 cm (Marcus et al., 1961b).

Oxygen uptake studies were conducted by employing the Warburg constant volume respirometer technique (Umbreit, Burris, and Stauffer, 1957). The substrate was 2.5 ml of tryptose phosphate broth. Standardized amounts of bacteria were added in a volume of 0.5 ml. The center well contained 0.2 ml of 20 per cent KOH plus a 1 cm² fluted filter paper. Flasks were set up in duplicate.

RESULTS

Effect of Varying Temperatures

In order to assess the effects of acute and chronic low temperature stress on mechanisms of specific and nonspecific resistance to microorganisms, it became apparent that information concerning the behavior of the challenge agents at low temperatures was necessary. It has been observed that certain small animals undergo a considerable drop in core temperature when placed in an environment in which the ambient temperature is considerably less than the normal core temperature. Since specific information concerning the metabolic and growth behavior of the challenge agents (*K. pneumoniae* and *S. aureus*) at temperatures lower than 37° C was lacking, it became apparent that data were necessary for the interpretation of experiments involving specific and nonspecific resistance mechanisms at core temperatures less than normal.

Growth of the bacteria was determined by employing turbidity as a function of time. Turbidity of the growing cultures was measured in a Klett-Summerson photoelectric colorimeter with a blue filter. The numbers of organisms present for any given turbidity measurement was determined from a standard curve based on the assumption that each organism present divides at the same time; the generation time for these experiments is defined

SPECIFIC AND NONSPECIFIC RESISTANCE

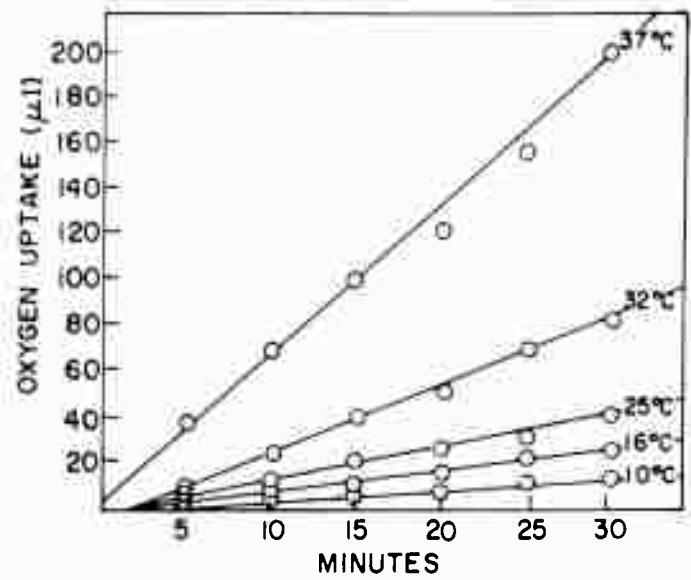


Figure 1. Effect of varying temperatures on oxygen uptake of *Klebsiella pneumoniae*.

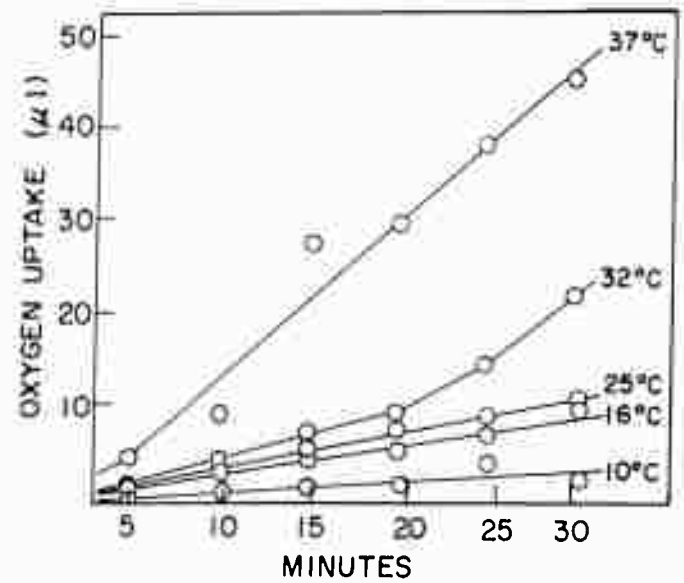


Figure 2. Effect of varying temperatures on oxygen uptake of *Staphylococcus pyogenes aureus*.

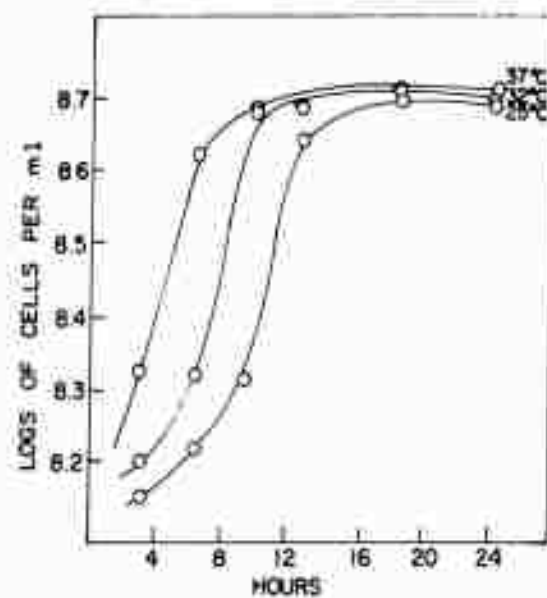


Figure 3. Effect of varying temperatures on growth of *Klebsiella pneumoniae*.

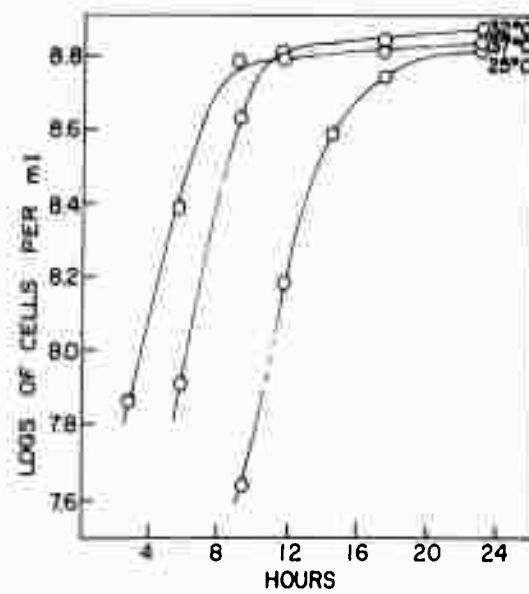


Figure 4. Effect of varying temperatures on growth of *Staphylococcus pyogenes aureus*.

SPECIFIC AND NONSPECIFIC RESISTANCE

as the time required for the numbers of bacteria present to double in population.

Constant temperature water baths which maintained the specified temperature $\pm 0.5^{\circ}$ C were employed in all the experiments.

Effect of varying temperatures on oxygen uptake. Figures 1 and 2 show that a number of different curves are obtained when oxygen uptake is plotted as a function of time for a given temperature. The results appear identical with the strains employed in K. pneumoniae and S. aureus. A significant decrease in oxygen uptake occurs at 32° C compared to that occurring at 37° C. At temperatures below 32° C, the oxygen uptake rates are again lessened, but little change is noted between 20° C and 25° C. It is of interest that the S. aureus oxygen uptake is significantly lower than that of K. pneumoniae when the same temperatures are compared.

Effect of varying temperatures on growth and generation times. Calibrated cuvettes (13 x 100 mm) containing 5 ml of tryptose phosphate broth were seeded with a standard loop (0.01 ml) of broth inoculum obtained from an 18-hour culture. The optical density was measured at zero time and thereafter at various increments of time. Duplicate sets of cuvettes were employed. The number of organisms for any given period of time was determined by reference to a standard curve. The results are shown in Figures 3 and 4.

It is seen that similar results were obtained with the strains of K. pneumoniae and S. aureus. In both cases, families of curves were obtained. The slopes of the curves are not significantly different from each other, indicating that the generation time at temperatures ranging from 37° C to 25° C are not markedly affected by different temperatures, but a noticeable increase in the lag phase is seen. No measurable increase in turbidity occurred at temperatures below 25° C. It is possible that, for the time employed, multiplication did not occur or was too slow to be measured by this technique.

Studies on Klebsiella Pneumoniae Passed Through Mice Maintained at Low Ambient Temperatures

The previous section revealed information concerning the metabolic behavior of the bacterial challenge agents at different temperatures; however, it was not possible to predict the metabolic behavior and virulence of the organisms when given to an animal that was acclimatized to the cold. In order to see if any changes did occur, the following experiment was carried out.

A single isolated colony of *K. pneumoniae* was inoculated into tryptose phosphate broth (Difco) and incubated for 18 hours at 37° C. One tenth ml of broth suspension of organisms per mouse was given intraperitoneally. At the end of 24 hours, the surviving mice were sacrificed by decapitation. The peritoneal cavities were opened aseptically and the peritoneal exudate was removed. The exudate was reinjected into a number of mice after a sample had been seeded to heart infusion blood agar plates (Difco) for isolation and identification procedures. This procedure was repeated each day for 7 days. The inoculated mice were kept at either 21° C or 2° C during this time of organism passage. The mice had been kept at these respective temperatures for 40 days prior to injection with the organisms. The *K. pneumoniae* strain isolated from the animals kept at 2° C was incubated at room temperature (21° C) while the organisms isolated from the animals kept at 21° C were incubated at 37° C.

The *K. pneumoniae* passed through mice maintained at 2° C was compared with the *K. pneumoniae* passed through mice maintained at 21° C with respect to their metabolic behavior and virulence. It is seen in Figure 5 that incubation of both strains at 37° C results in similar growth behavior curves when comparing organisms isolated from the animals maintained at 2° C or at 21° C. Slightly different growth curves in terms of comparison of the two strains were obtained when these organisms were incubated at 32° C (Figure 6). The *K. pneumoniae* isolated from animals maintained at 2° C showed growth curves almost identical with those obtained at 37° C incubation, whereas the organisms isolated from mice maintained at 21° C show a definite decrease in growth maximum in addition to an increased lag phase. These results suggested that

SPECIFIC AND NONSPECIFIC RESISTANCE

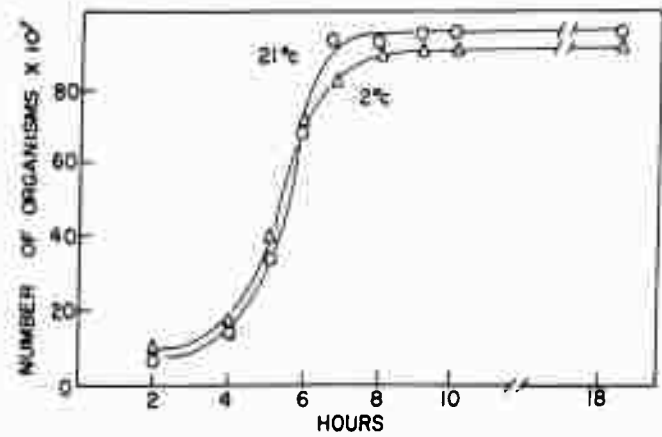


Figure 5. Effect of 37° C incubation temperature on growth behavior of *K. pneumoniae* isolated from mice maintained at 2° C and 21° C.

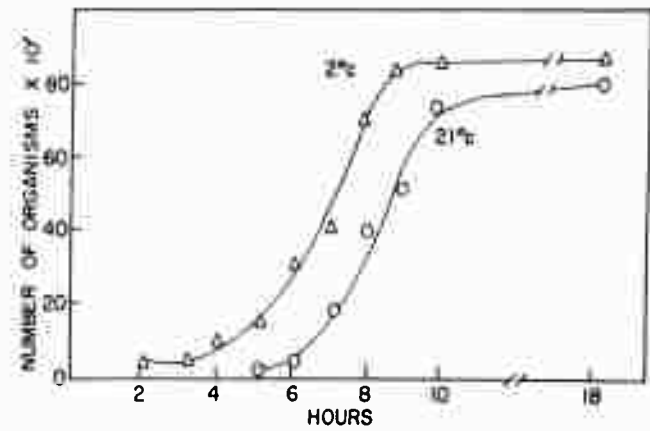


Figure 6. Effect of 32° C incubation temperature on growth behavior of *K. pneumoniae* isolated from mice maintained at 2° C and 21° C.

MIYA, MARCUS AND PHELPS

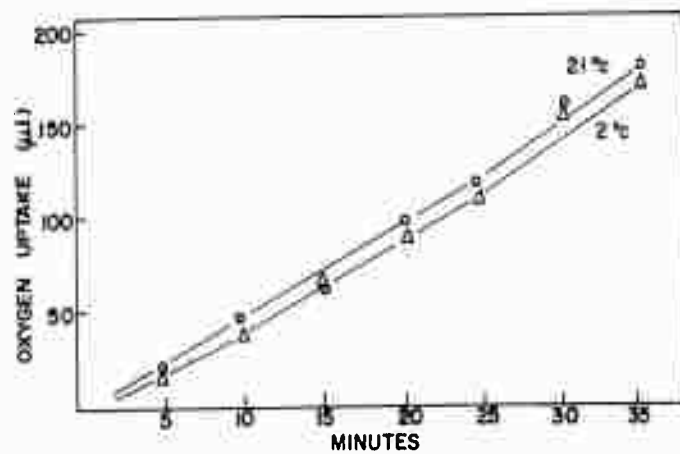


Figure 7. Effect of 37° C incubation temperature on oxygen uptake of *K. pneumoniae* isolated from mice maintained at 2° C and 21° C.

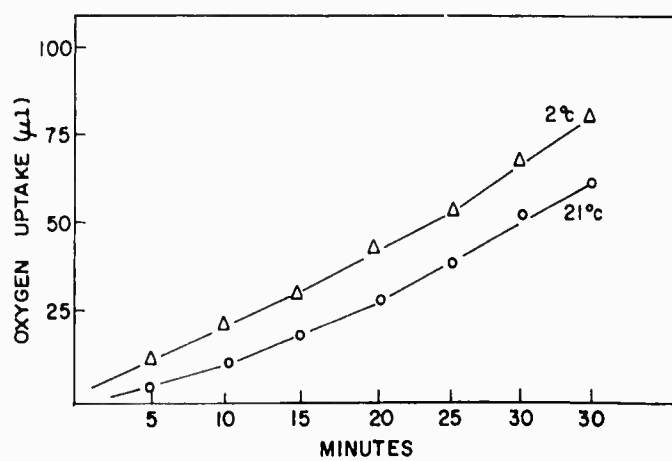


Figure 8. Effect of 32° C incubation temperature on oxygen uptake of *K. pneumoniae* isolated from mice maintained at 2° C.

SPECIFIC AND NONSPECIFIC RESISTANCE

an organism isolated from a low temperature acclimatized animal can grow equally well at the optimal temperature of 37°C or at reduced incubation temperatures of 32°C , whereas the organism isolated from an animal kept at 21°C does not have this dual growth capacity.

The situation becomes more confusing when one looks at the oxygen uptake curves. When both the isolated strains of organisms are incubated at 37°C , the oxygen uptake curves are similar qualitatively and quantitatively (Figure 7). When both isolated strains of organisms were incubated at 32°C , the oxygen uptake of the *K. pneumoniae* isolated from the low temperature acclimatized animals was greater than that of the organisms isolated from mice maintained at 21°C . This is what one would expect since the growth curves exhibit these same changes; however, note that quantitatively, the amount of oxygen uptake for both isolates is approximately one-half when incubation is carried out at 32°C compared to 37°C . This implies that the isolate from the mice kept at 21°C is more efficient metabolically since it can grow as rapidly at 32°C as it can at 37°C , yet requires only one-half the amount of oxygen to do so (Fig. 8).

The LD_{50} values of both isolates are summarized in Table I. No significant differences or changes in the virulence of the organisms were observed.

Mouse temperature measurements. In order to determine the effect huddling had on mouse temperature measurements, the following experiment was conducted. Mice were placed at 21°C or 2°C in cages containing either a single mouse or a group of five mice. Core, skin, and upper respiratory cavity temperatures were taken hourly for the first four to five hours on the first day, then once a day for 8 to 14 days, and finally on the forty-fifth day of exposure. Figure 9 shows that the presence of five mice in one cage at 2°C results in temperature measurements that increase gradually, reaching an initial maximum in 2 to 4 hours after exposure. Generally, the rectal temperature is consistently higher than the upper respiratory cavity, but it does not appear to be significantly greater. The skin temperatures are considerably less than the rectal or upper respiratory cavity temperatures, so the

MIYA, MARCUS AND PHELPS

Temperature of Experiment	Dose	Organism	Mortality Ratio	LD ₅₀ (95% Confidence Limits)
2° C	1040	P	10/10	20 (14.1 - 28.4)
	104		9/10	
	10.4		4/10	
	1.04		0/10	
2° C	1040	NP	10/10	35 (14.6 - 84.0)
	104		8/10	
	10.4		3/10	
	1.04		0/10	
21° C	1040	P	10/10	35 (15.9 - 77.0)
	104		7/10	
	10.4		4/10	
	1.04		0/10	
21° C	1040	NP	10/10	35 (3.5 - 350)
	104		4/10	
	10.4		5/10	
	1.04		0/10	

Table 1. LD₅₀ values of *Klebsiella pneumoniae* isolated from mice maintained at 2° C(P) and 21° C(NP) following intraperitoneal injection.

three temperature curves appear to parallel each other.

In Figure 10 are presented the results of temperature measurements obtained on singly-caged mice maintained at 2° C. Again the three temperature curves parallel each other, and the temperature difference is of the following descending order: rectal, upper respiratory cavity, skin. The rectal temperatures are not significantly greater than the upper respiratory cavity temperatures, but they do show consistently higher values. Similar to the results obtained with the grouped mice, the skin temperatures of the singly-caged mice are considerably lower than the rectal and upper respiratory cavity temperatures. It is of interest to note that an initial rise in temperature occurs within one hour after exposure to the low ambient environment, and is then followed by a sharp drop in temperatures which reaches a maximal fall by four hours post-exposure. From this point the temperatures gradually rise to reach stability by 24 hours post-exposure. The time of stabilization appears to be the same as that required for the grouped mice.

SPECIFIC AND NONSPECIFIC RESISTANCE

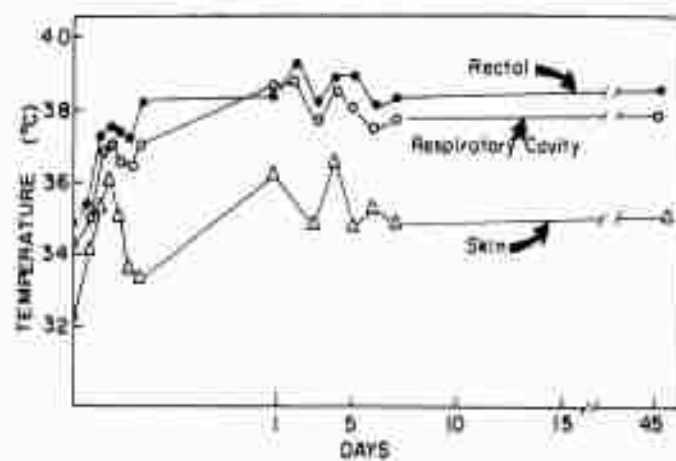


Figure 9. Effect of grouping (5 mice/group) on average temperatures of mice maintained at $2^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

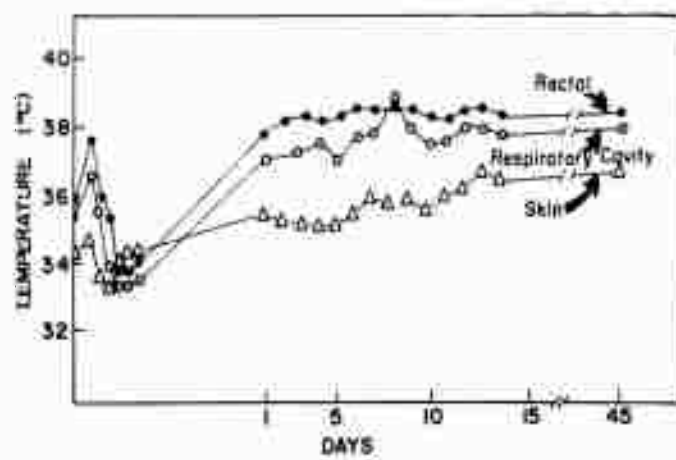


Figure 10. Effect of single caging on average temperatures of mice maintained at $2^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (average of 10 mice).

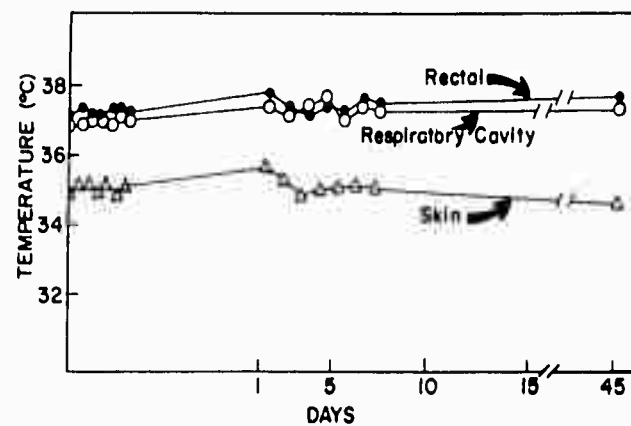


Figure 11. Effect of grouping (5 mice/group) on average temperatures of mice maintained at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

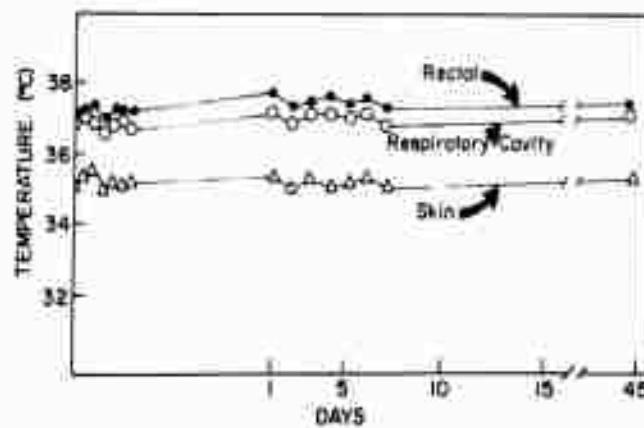


Figure 12. Effect of single caging on average temperatures of mice maintained at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (average of 10 mice).

SPECIFIC AND NONSPECIFIC RESISTANCE

Number of Organisms	Ambient Temperatures					
	21° C (grouped)		2° C (grouped)		2° C (single)	
	normal	immu- nized	normal	immu- nized	normal	immu- nized
4.3	8/10*	0/10	7/8	0/10	8/8	6/10
43	9/10	0/10	9/10	0/10	9/10	7/10
430	10/10	0/10	10/10	0/8	10/10	6/10
4300	10/10	0/10	10/10	1/8	10/10	6/10
Agglutinin titer**	0	1:32	0	1:32	0	1:32

Table II. Effect of acute cold stress (2° C) on mortality of mice challenged with *Klebsiella pneumoniae*. Unacclimatized animals injected at room temperature, then placed at noted temperatures. *7 day mortality (dead/total). **Titer before challenge.

When temperatures are measured on mice grouped five per cage and maintained at 21° C, the results shown in Figure 11 are obtained. The rectal and upper respiratory cavity temperatures are about the same in magnitude, while the skin temperatures range about 2° C to 2.5° C less. The temperature curves again parallel each other, but they do not exhibit any marked fluctuations as seen with mice maintained singly or grouped at 2° C. Essentially, the temperatures of the mice remained quite constant throughout the experiment. The data charted in Figure 12 demonstrate that mice singly caged and kept at 21° C exhibited temperature curves that were almost identical with those obtained with grouped mice maintained at the same ambient temperature.

Effect of Acute and Chronic Exposure to Low Temperatures on Survival of Mice Challenged with *Klebsiella Pneumoniae*

Acute exposure experiments. Mice were immunized at room temperature and then were challenged with varying numbers of *K. pneumoniae*. Immediately following this procedure, the mice were transferred to an ambient temperature of 2° C. The con-

MIYA, MARCUS AND PHELPS

Number of Organisms	Ambient Temperatures					
	21° C (grouped)		2° C (grouped)		2° C (single)	
	normal	immu- nized	normal	immu- nized	normal	immu- nized
5,6	3/10*	0/10	10/10	0/10	7/9	0/10
56	4/10	0/10	9/9	1/10	7/8	0/10
560	10/10	0/10	10/10	2/10	6/6	1/10
5600	10/10	0/10	10/10	2/10	9/9	1/10
LD ₅₀ (95% Con- fidence limits)	56 (52-59)	-	-	51 x 10 ⁴ (49 x 10 ⁴ - 52 x 10 ⁴)	4.1 (3.8- 4.4)	
Agglutinin titer**	0	1:32	0	1:16	0	1:16

Table III. Effect of chronic cold stress (2° C) on mortality of mice challenged with *Klebsiella pneumoniae*. Animals maintained for 45 days, and immunized at noted temperatures. *7 day mortality (dead/total).

trol groups of mice were kept at 21° C. The results in Table II show that immunization protected the grouped animals. In contrast, the immunization procedure was less effective in mice singly-caged. The nonchallenged stress controls placed at 2° C did not die when grouped, but some (10 per cent) did when singly caged, suggesting that huddling of animals enables a more favorable outcome when animals are acutely stressed by low ambient temperatures.

Chronic exposure experiments. Two groups of mice were placed at 2° C and 21° C for periods of 45 days before being immunized. One week after the last immunizing injection, the mice were challenged with varying numbers of viable *K. pneumoniae*. The results in Table III show that mice were significantly protected by the immunization procedure. The titer of agglutinin antibody formed by mice chronically exposed to 2° C was comparable to that formed by animals kept at 21° C. It is interesting that no significant differences in

SPECIFIC AND NONSPECIFIC RESISTANCE

mortality of grouped or singly-caged normal animals was observed. Immunization protected the counterpart groups to the same extent. It can be concluded from these results that the mice chronically exposed to an ambient temperature of 2° C were able to form agglutinin antibody and that the immunization procedure offered significant protection against the challenge organisms. In contrast, the normal mice chronically exposed to 2° C were adversely affected by *K. pneumoniae*; that is, smaller numbers of organisms caused increased mortality whether the animals were grouped or caged individually.

Effect of Acute and Chronic Low Temperature Stress on Survival of Mice Challenged with Staphylococcus Aureus

Four strains of *S. aureus* were tested for their virulence for mice via the IP route in order to determine which would be the most suitable for subsequent studies. The Fritchie strain was found to be the most virulent. The LD₅₀ was 25×10^7 organisms with 95 per cent confidence limits of 13×10^7 to 47×10^7 . Deaths of animals challenged with one LD₅₀ of the Fritchie strain usually occurred within 5 to 10 hours after challenge; however, the experiments were not terminated until 7 to 10 days had elapsed.

In the acute exposure experiments, mice were immunized at 21° C. Immediately following the IP challenge, the mice were kept either at 21° C or transferred to 2° C. The mice that were transferred to the low ambient temperature were caged either in groups of 10 animals or as individuals. The results of a typical experiment in Table IV show that a dose-response effect from *S. aureus*, Fritchie strain, is obtainable whether mice are exposed to the cold environment or kept at room temperature. Note that immunization is effective in protecting the challenged mice kept at 21° C and in groups at 2° C; in contrast, immunization did not afford protection to mice caged individually. Although not listed in the table, non-challenged cold stress control mice caged individually did not die during the experimental period (mortality ratio, 0/10).

Mice that were chronically exposed to low ambient temperatures

MIYA, MARCUS AND PHELPS

Number of Organisms	Ambient Temperature					
	21° C		2° C		2° C	
	(grouped) normal	immu- nized	(grouped) normal	immu- nized	(single) normal	immu- nized
1.67×10^9	10/10*	2/10	10/10	1/10	10/10	8/10
1.67×10^8	6/10	0/10	10/10	0/10	10/10	7/10
1.67×10^7	0/10	0/10	0/10	0/10	4/10	1/10
1.67×10^6	0/10	0/10	0/10	0/10	0/10	0/10
LD ₅₀ and 95% Confidence Limits	10×10^7 (4.9×10^7 to 22×10^7)	-	-	-	3.5×10^7 (1.2×10^7 to 9.8×10^7)	6.5×10^7 (1.7×10^7 to 24×10^7)
Agglutinin titer	0	1:32	0	1:32	0	1:32

Table IV. Effect of acute cold (2° C) on mortality of mice challenged with *Staphylococcus aureus*, strain Fritchle. Unacclimatized animals immunized and challenged at room temperature, then placed at noted temperatures. *7 day mortality (dead/total).

either in groups or individually were kept at this temperature for 21 days. These acclimatized mice were immunized at this temperature and were subsequently challenged one week after the last immunizing injection. Table V illustrates that the immunization of the animals kept at 21° C afforded protection to the challenged animals. However, immunized mice kept in groups or individually at 2° C did not obtain the benefits of immunization as well as the animals kept at 21° C. In addition, the singly caged animals at 2° C showed even less benefit from immunization than did the grouped counterpart animals kept at the same low temperature. As with the *K. pneumoniae* experiments, agglutinin antibody formation was not impaired in mice chronically exposed to low ambient temperatures, whether the animals were caged in groups or individually. In contrast to the *K. pneumoniae*

SPECIFIC AND NONSPECIFIC RESISTANCE

Number of Organisms	Ambient Temperatures					
	21° C		2° C		2° C	
	(grouped)	immu- nized	(grouped)	immu- nized	(single)	immu- nized
	normal		normal		normal	
1.64×10^9	7/8*	4/10	10/10	6/10	10/10	10/10
1.64×10^8	7/8	0/10	5/10	4/10	6/10	7/10
1.64×10^7	1/6	0/10	4/10	2/10	3/10	3/10
1.64×10^6	0/7	0/10	1/10	2/10	1/10	1/10
LD ₅₀ and	20×10^6		50×10^6	100×10^6	25×10^6	16×10^6
95%	(2.0×10^6)		(11×10^6)	(25×10^6)	(5.5×10^6)	(3.2×10^6)
Confidence	to		to	to	to	to
Limits	200×10^6		225×10^6	400×10^6	112×10^6	80×10^6
Agglutinin titer	0	1:32	0	1:16	0	1:16

Table V. Effect of chronic cold stress (2° C) on mortality of mice challenged with *Staphylococcus aureus*, strain Fritchle. Animals maintained for 21 days, immunized and challenged at noted temperatures. *7 day mortality (dead/total).

experiments, acclimatized and immunized mice, singly-caged or in groups, did no better on challenge with *S. aureus* than controls.

Low Ambient Temperatures and Specific and Nonspecific Resistance

Effect of acute exposure following one LD₅₀ challenge dose. The animals in this experiment were maintained at 21° C during the time of specific immunization or treatment with zymosan or endotoxin. Following challenge with viable organisms, some of the animals were immediately transferred to 2° C; others were kept at 21° C as controls. The mice that were transferred to the cold room were either kept in groups of 10 or caged indi-

MIYA, MARCUS AND PHELPS

Temperature	Treatment	Mortality ratios at 14 days (grouped)	(single)
21° C	Normal	8/10	-
	Immune	1/10	-
	Zymosan	6/10	-
	Endotoxin	9/10	-
2° C	Normal	10/10	8/10
	Immune	1/10	4/10
	Zymosan	8/10	9/10
	Endotoxin	7/10	9/10
	-	0/10	1/10

Table VI. Effect of acute cold (2° C) on mortality of mice challenged intraperitoneally with 1 LD₅₀* of Staphylococcus aureus, strain Fritchie. Unacclimatized animals immunized or treated and challenged at room temperature, then placed at noted temperatures. *1 LD₅₀ of S. aureus = 75 x 10⁶ organism per mouse as determined by method of Litchfield and Wilcoxon (1949).

vidually. The mortality ratios that were obtained under these conditions are shown in Tables VI and VII.

Table VI shows that mice challenged with 1 LD₅₀ of S. aureus are afforded protection when specifically immunized and kept in groups of ten while acutely exposed to 2° C. However, mice caged as individuals are not afforded the same degree of protection, although resistance greater than that of normal mice is evident. The nontreated acutely stressed control mice did not die when grouped, and only 10 per cent (1/10) died when caged singly. Therefore, most of the deaths that occurred may be attributed primarily to the challenge of organisms. Mice receiving zymosan or endotoxin treatment prior to challenge did not fare as well as the specifically immunized animals, and the significance of the difference in mortality ratios between the normal and nonspecifically immunized

SPECIFIC AND NONSPECIFIC RESISTANCE

Temperature	Treatment	Mortality ratios at 14 days	
		(grouped)	(single)
21° C	Normal	3/10	-
	Immune	0/10	-
	Zymosan	0/10	-
	Endotoxin	2/9	-
2° C	Normal	3/10	2/10
	Immune	0/10	4/10
	Zymosan	3/10	4/10
	Endotoxin	4/10	7/10
	-	0/10	1/10

Table VII. Effect of acute cold (2° C) on mortality of mice challenged intraperitoneally with 1 LD₅₀* of *Klebsiella pneumoniae*. Unacclimatized animals immunized or treated and challenged at room temperature, then placed at noted temperatures. *1 LD₅₀ of *K. pneumoniae* = 68 organisms per mouse as determined by method of Litchfield and Wilcoxon (1949).

mice is not significant. It should be mentioned here that mice kept at 21° C in groups or caged singly did not show any differences in mortality ratios as determined by preliminary experiments, and therefore the results obtained with the mice kept at 21° C in groups can serve as controls for the mice kept as individuals at 2° C.

The results presented in Table VII summarize the mortality ratios obtained when mice are subjected to acute cold stress following challenge with 1 LD₅₀ of *K. pneumoniae*. Again, specific immunization afforded the best protection against the induced infection when mice were subjected to acute cold stress. However, the mice kept in groups were better protected than mice caged individually and subjected to low ambient temperatures. The mice treated with zymosan and kept at 21° C showed no mortality, but the mice receiving the same treatment and placed at 2° C, whether

MIYA, MARCUS AND PHELPS

Temperature	Treatment	Mortality ratios at 14 days	
		(grouped)	(single)
21° C	Normal	8/10	-
	Immune	1/10	-
	Zymosan	6/10	-
	Endotoxin	9/10	-
2° C	Normal	9/10	10/10
	Immune	0/10	2/10
	Zymosan	6/10	8/10
	Endotoxin	7/10	7/10
	-	0/10	0/10

Table VIII. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 1 LD₅₀* of *Staphylococcus aureus*, strain Fritchie. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. *1 LD₅₀ of *S. aureus* = 75 x 10⁶ organisms per mouse.

in groups or singly, were not afforded this protection. The results obtained with endotoxin are equivocal. The mortality ratio of the normal animals kept at 21° C was only 30 per cent (3/10). Results to be presented later in this paper deal with mortality ratios obtained when the challenge dose was increased to 10 LD₅₀.

Effect of chronic exposure following one LD₅₀ dose. The animals in this experiment were maintained at 2° C for 30 days in order to allow acclimatization to the low ambient temperature. The animals were specifically immunized or treated with zymosan or endotoxin at this low ambient temperature. Following these procedures the animals were challenged with one LD₅₀ dose of *S. aureus* or *K. pneumoniae*. The results are shown in Tables VIII and IX.

The animals receiving the *S. aureus* challenge (Table VIII) were protected if they were specifically immunized. Such protection occurred without regard to whether the mice were caged in groups

SPECIFIC AND NONSPECIFIC RESISTANCE

Temperatures	Treatment	Mortality ratios at 14 days (grouped)	(single)
21° C	Normal	3/10	-
	Immune	0/10	-
	Zymosan	0/10	-
	Endotoxin	2/9	-
2° C	Normal	4/10	6/10
	Immune	0/10	2/10
	Zymosan	0/10	9/10
	Endotoxin	9/10	7/10
	-	0/10	0/10

Table IX. Effect of chronic cold stress (2° C) on mortality of mice challenged intra-peritoneally with 1 LD₅₀* of *Klebsiella pneumoniae*. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. *1 LD₅₀ of *K. pneumoniae* = 68 organisms per mouse.

or as individuals. The nonspecific immunization treatment did not result in significant protection compared to the controls or to the specifically immunized animals. The mortality ratio of the zymosan treated animals compared to the endotoxin treated animals is not significantly different. When comparing mortality results of animals that are acclimatized versus the acutely cold stressed animals (Table VI vs. Table VIII), note that the acclimatized animals that are specifically immunized did well grouped or caged individually. In contrast, the acutely cold stressed singly-caged mice were not benefited by specific immunization to the extent noted for the acutely stressed mice caged in groups.

When the cold acclimatized mice were challenged with one LD₅₀ of *K. pneumoniae* (Table IX), both specific immunization and zymosan treatment were beneficial to animals kept at 21° C or in groups at 2° C. However, protective effects of endotoxin were not apparent under these conditions. When animals that have been

MIYA, MARCUS AND PHELPS

Temperature	Treatment	Mortality ratios at 14 days (grouped)
21° C	Normal	10/10
	Immune	0/10
	Zymosan	9/10
	Endotoxin	10/10
2° C	Normal	10/10
	Immune	0/10
	Zymosan	9/10
	Endotoxin	10/10
	-	0/10

Table X. Effect of acute cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD₅₀* of *Staphylococcus aureus*, strain Fritchie. Unacclimatized animals immunized or treated and challenged at room temperature, then placed at noted temperatures. *10 LD₅₀ of *S. aureus* = 50×10^8 organisms per mouse.

acutely cold stressed are compared to acclimatized animals, the latter do better than the former following challenge. It appears that zymosan may benefit the challenged host in the animals kept in groups under conditions of chronic cold stress, but not under conditions of acute cold stress. This protective effect of zymosan is not apparent in acclimatized animals caged singly. Again, some reservation in conclusions is warranted because of the low mortality ratio of the mice kept at 21° C following the one LD₅₀ challenge dose.

Effects of acute exposure following larger challenge dose. The specifically or nonspecifically treated animals in this experiment were maintained and challenged at room temperature. Following this, the animals were immediately transferred to 2° C or kept at 21° C as controls. The challenge dose was increased by several orders of magnitude over that in the previous experiments in order to obtain more definitive results concerning the efficacy of nonspecific versus specific resistance to disease. The animals were caged in groups of

SPECIFIC AND NONSPECIFIC RESISTANCE

Temperature	Treatment	Mortality ratios at 14 days (grouped)	P(x ²)
21° C	Normal	15/20	
	Immune	0/20	
	Zymosan	8/20	
	Endotoxin	11/19	
2° C	Normal	17/20	
	Immune	0/20	
	Zymosan	9/20	
	Endotoxin	14/20	
	-	0/10	

Table XI. Effect of acute cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD₅₀* of *Klebsiella pneumoniae*. Unacclimatized animals immunized or treated and challenged at room temperatures, then placed at noted temperatures.
*10 LD₅₀ = 1000 organisms per mouse.

ten. The results are shown in Tables X and XI. Table X shows that mice specifically immunized to the challenge agent, *S. aureus*, were significantly protected even though acutely exposed to 2° C. The mortality ratios of mice treated with zymosan or endotoxin prior to challenge was considerably increased over the specifically immunized group. The results indicate that the increased resistance afforded mice by specific immunization was not depressed by acute exposure of the animals to 2° C. The resistance induced, then, appeared to be temperature independent. There were no singly-caged animals in this experiment, since it was apparent from prior results that grouping afforded maximal protection even in groups with as few as three animals.

In Table XI are summarized the results of acute cold stress of specifically and nonspecifically immunized animals challenged with ten LD₅₀'s of *K. pneumoniae*. By increasing both the animal group size and challenge inoculum dose, we felt that the data obtained would

MIYA, MARCUS AND PHELPS

Temperature	Treatment	Mortality ratios at 14 days (grouped)
21° C	Normal	10/10
	Immune	0/10
	Zymosan	9/10
	Endotoxin	10/10
2° C	Normal	10/10
	Immune	1/10
	Zymosan	10/10
	Endotoxin	10/10
	-	0/10

Table XII. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD₅₀* of *Staphylococcus aureus*, strain Fritchie. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. *10 LD₅₀ of *S. aureus* = 50×10^8 organisms per mouse.

represent definitive results concerning whether nonspecific immunization procedures were as efficacious as specific immunization procedures. And once more, specific immunization offered the greatest resistance to experimental disease. This increased resistance was not affected by acute exposure to 2° C. The extent of protection afforded the animals by treatment with zymosan or endotoxin did not approach that afforded by specific immunization. By comparing the mortality ratio of the control animals with that of the zymosan treated animals, a probability value of significance is obtained. This significant degree of protection was independent of acute cold exposure. No significant differences in mortality ratios were noted between control animals and endotoxin treated animals.

Effect of chronic exposure following larger doses. Animals in this experiment were maintained at 2° C for 30 days to allow acclimatization. The animals were specifically or nonspecifically immunized at this low ambient temperature, and were caged in groups of 10. The challenge dose was increased to several LD₅₀'s in order to obtain

SPECIFIC AND NONSPECIFIC RESISTANCE

Temperature	Treatment	Mortality ratios at 14 days (grouped)
21° C	Normal	10/10
	Immune	0/10
	Zymosan	3/10
	Endotoxin	8/10
2° C	Normal	10/10
	Immune	0/10
	Zymosan	1/10
	Endotoxin	10/10
	-	0/10

Table XIII. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD₅₀* of *Klebsiella pneumoniae*. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. *10 LD₅₀ of *K. pneumoniae* = 1000 organisms per mouse.

more definitive results concerning the efficacy of specific and non-specific immunization under these conditions. Table XII shows the results obtained for animals challenged with *S. aureus*, and it is evident that chronic cold stress did not interfere with specific immune processes. Specifically immunized animals were significantly protected against parenterally induced disease even when exposed to challenge doses as high as 10 LD₅₀'s. There was no protection afforded the animals pretreated with zymosan or endotoxin. It should be mentioned here that the strain of *S. aureus* employed caused the deaths of the animals within 24 hours post-challenge. Those animals surviving the first 24 hours usually did not die subsequently. Therefore, the experiments were terminated at 14 days.

Table XIII shows the results of chronically cold exposed animals challenged with *K. pneumoniae*. Again, as noted in experiments already described, the specifically immunized animals were significantly protected against the ten LD₅₀ challenge. Endotoxin treatment yielded no protection to the mice. In contrast, the zymosan treated

MIYA, MARCUS AND PHELPS

animals had only 1 death in 10 at 2° C and only 3 deaths in 10 at 21° C. The results obtained with zymosan pretreatment are quite similar to those obtained with mice acutely cold stressed following challenge with *K. pneumoniae*. This would suggest that the mechanisms whereby zymosan acts to increase resistance to experimental disease is independent of acute or chronic exposure at 2° C.

Viruses

Coxsackie virus infections are manifest in different organ systems in infant mice, but not in adult mice (Dalldorf, 1950; Pappenheimer, Kunz, and Richardson, 1951; Boring, Angevine, and Walker, 1955). Since the morbidity and mortality properties of the Coxsackie strains obtained for these experiments was not known, preliminary experiments were designed to select the strain most suitable for experiments with adult mice, and the results of this screening procedure are summarized in Table XIV, which shows that challenge with Type B-3 Coxsackie virus caused mortality whether mice were kept at 2° C or 21° C. The University of Utah Type B-1 strain caused 4 deaths in 4 at 2° C and 1 death in 4 at 21° C, whereas the Connecticut 5 strain of Type B-1 caused 2 deaths in 4 at 2° C and zero deaths in 4 at 21° C. Since the experimental program as planned required a viral agent that would cause death of challenged animals at one temperature but not at the other, it was decided that the Type B-1 virus strain would be further screened with respect to mortality enhancement. The results shown in Table XV point out that neither viral agent caused mortality in mice kept at 21° C; however, a significant difference in mortality ratios is seen in mice kept at 2° C. The University of Utah Type B-1 Coxsackie virus strain was chosen as the subsequent experimental agent.

In the first definitive experiment, mice were acclimatized at 2° C for 40 days prior to challenge. The animals were to be compared with unacclimatized animals and control animals kept at 21° C. The mice were randomly segregated into groups of ten prior to challenge. Mice were pretreated with either zymosan or endotoxin. In addition a third group was given formalin killed virus 7 days prior to challenge. Unacclimatized mice were kept, treated, challenged at 21° C, and then placed at 2° C. The protocol and results are

SPECIFIC AND NONSPECIFIC RESISTANCE

Virus Strain	Mortality ratios at 14 days***	
	2° C	21° C
B-1*	4/4	1/4
B-2*	1/4	0/4
B-3*	4/4	3/4
B-4*	1/4	0/4
B-5*	1/4	0/4
B-1 (Conn. 5)**	2/4	0/4
Cold Control	0/4	-

Table XIV. Comparison of different Type B Coxsackie virus strains on mortality production in unacclimatized adult mice. *University of Utah, Department of Microbiology Stock Strains. **Obtained from Dr. D. L. Walker. ***All challenge doses approximately 5×10^4 PFU i. p.

Virus	Mortality ratios at 14 days*	
	2° C	21° C
University of Utah, B-1	19/20	0/20
Conn. 5, B-1	9/20	0/20

Table XV. Mortality ratios of unacclimatized adult mice challenged with Type B-1 Coxsackie virus. *Challenge dose = 5×10^4 PFU i. p.

MIYA, MARCUS AND PHELPS

Temperature	Treatment	Mortality ratios at 7 days
21° C	Normal	0/10
	Immunized	0/10
	Zymosan	0/10
	Endotoxin	1/9
2° C (Unacclima- tized)	Normal	7/10
	Immunized	0/10
	Zymosan	3/10
	Endotoxin	8/8
	Cold Control	0/10
2° C (Acclimatized)	Normal	0/10
	Immunized	0/10
	Zymosan	2/10
	Endotoxin	3/10
	Cold Control	0/10

Table XVI. Effect of specific and nonspecific immunization on resistance of adult mice challenged with Type B-1 Coxsackie virus intraperitoneally. Challenge = 10×10^4 PFU.

summarized in Table XVI. The mortality ratios were taken only for 7 days post-challenge because the mice were inadvertently not fed one weekend, and most of the animals in the cold died, presumably from starvation. However, it is of interest to briefly analyze the results obtained. It is seen that the virus only caused one death in 39 mice kept at 21° C. This death occurred in the animals receiving endotoxin. It is also seen that unacclimatized mice did not fare as well as acclimatized mice and, in general, exposure to 2° C decreased the host's ability to withstand Coxsackie virus challenge. Of special interest is the indication that acclimatized mice seemed to withstand the Type B-1 Coxsackie virus challenge that was detrimental to unacclimatized mice, although specific immunization

SPECIFIC AND NONSPECIFIC RESISTANCE

Temperature	Treatment	Mortality ratios at 14 days
21° C	Normal	0/20
	Immunized	0/20
	Zymosan	0/20
	Endotoxin	3/20
2° C (Unacclimatized)	Normal	15/20
	Immunized	0/20
	Zymosan	8/20
	Endotoxin	20/20
	Cold Control	0/20
2° C (Acclimatized)	Normal	1/20
	Immunized	0/20
	Zymosan	4/20
	Endotoxin	7/20
	Cold Control	0/20

Table XVII, Coxsackie B-1 infection mortality in adult mice. Challenge = 10×10^4 PFU intraperitoneally.

procedures protected the animals under these conditions. With the doses and routes of administration employed in this experiment, zymosan and endotoxin did not enhance host resistance to viral disease.

Although most deaths of animals in the preliminary experiment occurred between 4 and 7 days post-challenge and probably the results of the first definitive experiments were valid at 7 days, further investigation was required in order to see if acclimatization was beneficial to the challenged animals. Therefore, a repeat experiment of the same design was completed, but larger groups of mice were employed. The animals kept at 2° C were supplied with adequate amounts of food and water to rule out nutritional factors that might

MIYA, MARCUS AND PHELPS

confuse the analysis of data. The results of the experiment are shown in Table XVII. The majority of deaths occurred between 4 and 6 days post-challenge; no deaths of animals occurred after 10 days following challenge. Again, acclimatization at 20° C for 40 days resulted in mortality ratios similar to those observed in the animals kept at 21° C. Unacclimatized mice had increased mortality ratios in all treated groups compared to the 21° C control mice except in the immunized groups. Zymosan appeared to give greater protection than endotoxin; in fact, the 20 out of 20 deaths in the endotoxin group occurred by the fourth post-challenge day. If the mice were acclimatized and treated with endotoxin, only 7 in 20 deaths occurred. Thus, it would appear that acclimatization enhances host resistance to challenge in addition to decreasing the increased mortality ratios in the groups treated with endotoxin. The value of specific immunization is apparent in the unacclimatized mice.

Low Ambient Temperature and Ehrlich Ascites Tumor

The effect of temperature on neoplastic diseases has been studied by various investigators (Fay and Henny, 1938; Smith and Fay, 1939; Bischoff and Long, 1939; Wallace et al., 1944; Fuller et al., 1941; Goldfeder, 1941; Wallace et al., 1942; Tannenbaum and Silverstone, 1949; Griffiths et al., 1961). Effects on the tumor take, incidence of spontaneous tumor formation, growth, or regression were noted as a result of high or low ambient temperatures, but no consistent effects have been noted. In this respect, the effect of temperature on neoplastic disease becomes as complicated as that observed with regard to bacterial or viral disease.

Since the Ehrlich ascite tumor produces certain effects in mice similar to infectious disease, for example, rapidly progressive disease, it was chosen as a model to study the effect of cold stress on the disease process. Mice were exposed in the cold room in large animal cages containing 50 mice and acclimatized to this temperature for 45 days. Two days prior to challenge the mice were caged in groups of six. The mice were given unlimited amounts of Purina mouse chow and tap water. Mice kept at room temperature were randomly separated into groups of 12.

SPECIFIC AND NONSPECIFIC RESISTANCE

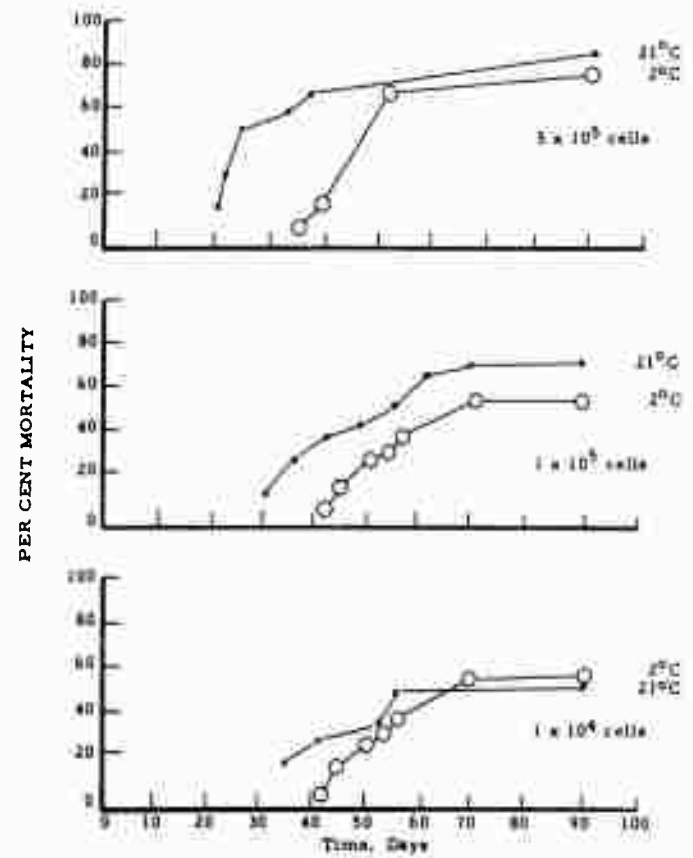


Figure 13. Mortality curves of acclimatized mice subcutaneously challenged with Ehrlich Ascites tumor cells and maintained at low ambient temperature (2° C).

MIYA, MARCUS AND PHELPS

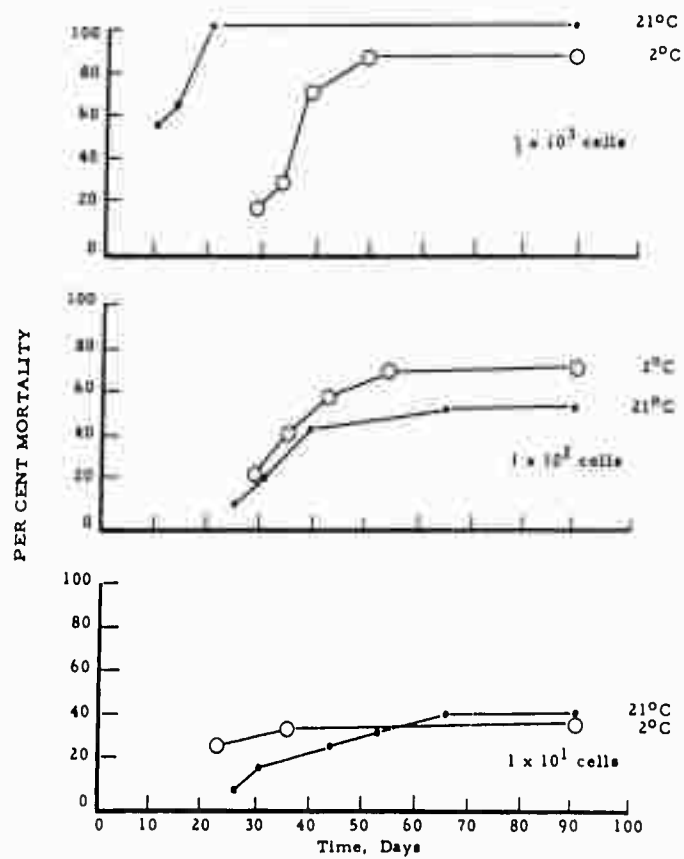


Figure 14. Mortality curves of acclimatized mice intraperitoneally challenged with Ehrlich Ascites tumor cells and maintained at low ambient temperature (2°C).

SPECIFIC AND NONSPECIFIC RESISTANCE

Route	Inoculation Dose	Mortality Ratios	
		2° C	21° C
S. C.*	10 ⁴	4/12***	6/12
	10 ⁵	7/12	9/12
	5 x 10 ⁵	9/12	9/12
I. P.**	10	4/12	6/12
	100	7/12	8/12
	1000	11/12	12/12

Table XVIII. Mortality ratio of acclimatized mice kept at 2° C and inoculated with Ehrlich Ascites tumor cells. *Subcutaneous Challenge. **Intraperitoneal Challenge. ***Dead/Total.

Ehrlich ascites tumor cells were kept in a stock suspension and occasionally were passed intraperitoneally into mice. The tumor cells were harvested and washed three times with Hank's balanced salt solution (BSS). The cells were suspended in Hank's BSS, and viable cells were counted in the hemocytometer using a 0.05 per cent solution of eosin as diluent. Stained cells were considered non-viable. The cells were then diluted to the desired concentration in the same medium.

Subcutaneous challenge. The challenge inocula were prepared as described and adjusted to contain 10⁴, 10⁵, and 5 x 10⁵ tumor cells per 0.5 ml. The cells were injected into the nuchal region. The experiment was terminated 90 days after inoculation of the cells. Low ambient temperatures as shown in Table XVIII did not significantly affect the final mortality ratios, regardless of the inoculum size. However, when animal mortality is plotted as a function of time, a significant delay in mortality is seen (Fig. 13). The effect is most pronounced in the highest challenge doses while a graded diminished effect is evident in the lower doses.

Intraperitoneal challenge. The challenge inocula were prepared as described and adjusted to contain 10, 100, and 1000 tumor cells per 0.5 ml. The acclimatized mice were challenged intraperitoneally,

MIYA, MARCUS AND PHELPS

and the mortality of the animals was followed for 90 days after challenge. The results in Table XVIII show that again low ambient temperatures did not influence the final mortality ratios regardless of challenge dose. However, when mortality is plotted as a function of time, a significant delay in mortality again is seen (Fig. 14). This delay in mortality is most pronounced in the animals receiving the highest challenge dose. At lower challenge doses, the mice died more rapidly when kept at 2° C than when kept at 21° C.

DISCUSSION

At the onset of this investigation, it was felt that knowledge of the metabolic behavior of the bacterial challenge agents was important in order to assess properly any subsequent data obtained when these agents were employed. The experimental results indicated that temperatures lower than 37° C affected the oxygen uptake but not the generation time of the organisms. The only apparent effect of lowered temperatures employed was to increase the lag phase.

Along with the above information, the necessity to determine the effect of low ambient temperatures on the body temperature of the mice used in our experimental set-up became apparent. As a first approximation, we assumed that if the body temperature changed as a result of cold exposure, then the change could directly influence the course of the experimental disease by enhancing or depressing the ability of the organisms to grow and develop. Our results showed that the rectal temperature of mice exposed to 2° C fluctuated within the first 24 hour period of cold exposure, but stabilized thereafter at a temperature 1° C to 2° C higher than mice kept at room temperature. The fluctuation was more pronounced in mice caged singly as compared to those caged in groups of five. In contrast, Walker and Boring (1958) showed that rectal temperatures of mice exposed to 4° C decreased approximately 1° C. The differences between results of the two groups reporting might be ascribed to a significant technical detail; that is, we in-

SPECIFIC AND NONSPECIFIC RESISTANCE

serted the probe uniformly 2 cm into the rectum, whereas Walker and Boring inserted the probe only 1 cm.

In spite of the differences in results of these two groups with regard to the increase or decrease in the body temperature of mice as a result of cold exposure, the degree of change was not outside the range compatible for optimal growth and development of the organisms employed for parenteral challenge. Therefore, we felt that any changes in susceptibility or resistance to disease could now be ascribed to factors other than that related to core temperature per se.

The fact that the K. pneumoniae isolated from animals maintained at 2° C did not vary in virulence as compared to the same organism isolated from mice maintained at 21° C led us to conclude that this organism could be used with confidence as a challenge agent to obtain the desired mortality ratios when given to mice kept at 21° C or 2° C. The observation that the isolate from mice maintained at 2° C would grow equally well at incubation temperatures of 37° C or 32° C requiring only one-half the oxygen used at 32° C suggests that these organisms are more metabolically efficient in contrast to organisms isolated from animals maintained at 21° C and grown under the same conditions. This deserves further investigation.

In general, specific immunization protected significantly, whereas nonspecific substances which have been used to increase resistance (zymosan and endotoxin) did not afford the same degree of protection when the challenged animals were exposed to 2° C either as acclimatized or unacclimatized mice. This was true whether the challenge agent was K. pneumoniae, S. aureus, or the B-1 strain of Coxsackie virus.

The increased resistance afforded by immunization was maximal when mice were exposed to low ambient temperatures in groups. Mice that were caged individually did not fare so well. Since the core temperatures of mice caged individually or in groups equilibrated within 24 hours of low temperature exposure, one cannot validly state that loss of body heat is a contributing factor to lessened resistance. However, it might be hypothesized that the metabolic rate of individually caged mice is increased over that of grouped animals,

MIYA, MARCUS AND PHELPS

since there is opportunity for greater heat loss and this increased metabolism is sufficient to result in the same end body temperature. Further, this increased metabolic rate resulting from the low temperature stress might eventually lead to exhaustion of body reserves and subsequent death (Selye, 1950).

Another possibility to account for variations in results is a psychological factor. Since immunized non-challenged mice are able to survive low ambient temperatures when caged individually but not when challenged with an infectious disease agent and maintained individually at low ambient temperatures, one must speculate concerning the extent that the factor of isolation contributes to increased mortality observed. Psychological factors cannot be disregarded in assessing this problem.

In some instances, zymosan or endotoxin treatment resulted in increased mortality ratios. This was noted consistently when the challenge agent was K. pneumoniae or the strain of Coxsackie virus, but not as apparent when S. aureus was used. This paradox, if real, requires further investigation, since nonspecific immunizing agents are known either to enhance or depress resistance depending on the time of administration; yet our observations suggest that the dosage and administration time optimal for a given ambient temperature may not be optimal for another ambient temperature.

The relationship of zymosan to properdin, shown to exist by Pillemer et al. (1956), deserves attention with regard to speculation concerning the extent the properdin system plays in the observed results with zymosan in our experiments. The dosage of 9 mgm subcutaneously is in excess of that reported by Ross (1956) to stimulate increases in properdin levels in mice. Ross injected the material intravenously, and the colloidal nature of zymosan certainly would limit the amount employed in order to avoid pulmonary embolic complications. Recently, Iakovleva and Remezov (1960) reported that mice exposed to the cold have greater than normal levels of properdin 72 hours post-exposure. The properdin level increasing action of zymosan added to the levels obtainable by cold exposure should insure mice of a high properdin level. However, since the serum of mice lacks complement components (Rice and Crowson, 1950) and has been shown to be devoid of bactericidal activity

SPECIFIC AND NONSPECIFIC RESISTANCE

(Marcus, Esplin and Donaldson, 1954), it seems unlikely that the properdin system is contributing much to the natural defenses of the mouse (Miya, Marcus, and Perkins, 1960).

Results of our experiments indicate that mice chronically exposed to cold are able to form agglutinin antibody. Trapani (1960) reported that cold exposed rabbits were able to form antibody almost as rapidly as rabbits kept at room temperature. In contrast, Ipsen (1952) reported that antibody formation is impaired in mice exposed to 4° C. We have not attempted to determine if antibody formation is impaired by acute exposure to cold.

Although the results of the Cocksackie virus experiments suggest that acclimatization will result in a normal degree of host resistance of nonimmune animals against challenge with this agent, the results of others are not in agreement. Walker and Boring (1958) reported on experiments using a Connecticut 5 strain of Type B-1 Cocksackie virus. They observed that acute limited exposure to 4° C was insufficient to change the viral infection from an asymptomatic into a lethal process, but that continued exposure for several days did accomplish conversion to disseminated lethal disease. They reported on experiments in which adapted (14 days) animals were employed, and found that this period of adaptation at 4° C did not counteract the lethal effects of the virus disease at 4° C. Mice challenged at 4° C, following acclimatization and then placed at 25° C, did not die. Therefore, Walker and Boring concluded that exposure to 4° C caused a decrease in resistance of the challenged animals.

In our experiments we have shown that acclimatization for 40 days at 2° C is sufficient for the adult mice to overcome a challenge dose that is lethal for virus challenged unacclimatized mice replaced in the cold box. Since the adrenal activity does initially increase upon cold exposure (Heroux and Hart, 1954; Schonbaum, 1960), and since the Cocksackie disease process resembled that due to cortisone effects, Walker and Boring (1958) attempted to reproduce the disease in mice at room temperature by ACTH injections following challenge with Cocksackie virus. They were unable to detect any decrease in resistance in mice treated in this manner. Although the increased mortality correlates well with increased corticosteroid

MIYA, MARCUS AND PHELPS

production following cold exposure, the exact cause and effect relationship remains obscure.

At present we are investigating the possibility that the virus may be present but inactive as a result of the acclimatization, but have the potential to cause disease or death if the challenged animal is removed from 2° C to 21° C. The situation could be analogous to that observed by Sulkin et al. (1960) with regard to bat rabies virus.

A brief discussion of the experiments with the mouse neoplasm is in order. Goldfeder (1941) reported that the environmental temperature has a definite effect on the growth rate but not the viability of subcutaneously inoculated tumor cells. Our results are similar in this respect. Although a delay in mortality occurred, no significant differences in the final mortality ratios were observed when mice exposed to cold were compared to mice challenged and kept at 21° C. An obvious working hypothesis is that the lowered skin temperature of mice kept at 2° C inhibited tumor cell metabolism until the tumor cells became acclimatized to the lower temperature. Once this occurred, it may be guessed, the growth rate of the acclimatized tumor cells was the same as for the controls.

The same temperature effect may be used to explain the delay in mortality of mice inoculated intraperitoneally; that is, the core temperature of 2° C exposed mice is about 2° C higher than mice kept at 21° C. This increased internal temperature may either alter tumor cell metabolism or allow for selection of tumor cells capable of growth at the new temperature. Work is continuing on this aspect of low ambient temperature and host resistance.

SPECIFIC AND NONSPECIFIC RESISTANCE

SUMMARY

In a series of experiments with mice, designed to explore the effect of low ambient temperatures on host-parasite relations, the following observations have been made:

1. The core temperature of mice exposed to 2° C stabilized within 24 hours 1° C to 2° C higher than that of mice maintained at 21° C.

2. The bacterial challenge agents, K. pneumoniae and S. aureus, were shown to be capable of growth at the core temperature fluctuations.

3. The K. pneumoniae employed was passed through and isolated from mice maintained at 2° C. This organism was equally capable of growth at incubation temperatures of 32° C or 37° C, although the oxygen requirements were one-half that required at 37° C.

4. Specific immunization resulted in the best protection when compared to zymosan or endotoxin treatment.

5. Zymosan and endotoxin treatment often resulted in increased mortality in mice exposed to 2° C and challenged with bacterial or viral agents.

6. Grouping of mice exposed to 2° C was significantly beneficial to survival following challenge as compared to singly-caged mice.

7. Acclimatization to 2° C resulted in equivocal protection to mice challenged with bacterial agents but was beneficial to mice challenged with a Cocksackie virus strain.

8. Low ambient temperatures influence the course of Ehrlich Ascites tumor disease process by retardation of mortality but do not affect the over-all mortality ratio.

MIYA, MARCUS AND PHELPS

LITERATURE CITED

1. Armstrong, C. 1938. Studies on mechanism of experimental intranasal infection in mice. Pub. Health Rep. 53: 2004-2012.
2. Armstrong, C. 1942. Some recent research in the field of neurotropic viruses with special reference to lymphocytic choriomeningitis and herpes simplex (Kober lecture). Mil. Surgeon 91: 129-146.
3. Bailey, A. L. 1960. The antibody response in rabbits to inactivated vaccines of type 6 ECHO virus and type 3 adenovirus. Master of Science Thesis, University of Utah.
4. Bischoff, F., and M. L. Long. 1939. The influence of low temperature environment on the growth of mouse sarcoma 180. Am. J. Cancer 35: 86-89.
5. Boring, W. D., M. Z. Rhem, and D. L. Walker. 1956. Factors influencing host-virus interactions. II. Alteration of Coxsackie virus infection in adult mice by cold. Proc. Soc. Exper. Biol. Med. 93: 273-277.
6. Bubel, H. C. 1958. The primary interaction of poliovirus with host cells of tissue culture origin. Doctor of Philosophy Thesis, University of Utah.
7. Dalldorf, G. 1950. The Coxsackie viruses. Bull. New York Acad. Med. 26: 329-335.
8. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with polioviruses. J. Exp. Med. 99: 167-182.
9. Fay, T., and G. C. Henny. 1938. Correlation of body segmental temperature and its relation to the location of carcinomatous metastasis. Surg. Gynec. and Obst. 66: 512-514.

SPECIFIC AND NONSPECIFIC RESISTANCE

10. Fuller, R. H., E. Brown, and C. A. Mills. 1941. Environmental temperatures and spontaneous tumors in mice. *Cancer Res.* 1: 130-133.
11. Goldfeder, A. 1941. The effects of reduced temperatures upon growth and metabolic changes of sarcoma 180 grown in vivo. *Cancer Res.* 1: 220-226.
12. Griffiths, J. D., E. Hoppe, and W. H. Cole. 1961. The influence of thermal stress and changes in body temperature on the development of carcinoma 256 Walker in rats after inoculation of cells. *Cancer* 14: 111-116.
13. Heroux, O., and J. S. Hart. 1954. Cold acclimation and adrenal cortical activity as measured by eosinophil levels. *Am. J. Physiol.* 178: 453-456.
14. Iakovleva, S. D., and P. I. Remezov. 1960. The properdin system under the influence of infection and various other unfavorable factors. *Zh. Microbiol. Epidemiol. Immunobiol.* 31: 7-12.
15. Ipsen, J., Jr. 1952. The effect of environmental temperature on the immune response of mice to tetanus toxoid. *J. Immunol.* 69: 273-283.
16. Lillie, R. D., R. E. Dyer, C. Armstrong, and J. G. Pasternack. 1937. Seasonal variation in intensity of brain reaction of St. Louis Encephalitis in mice and of endemic typhus in guinea pigs. *Pub. Health Rep.* 52: 1805-1822.
17. Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Therap.* 96: 99-113.
18. Marcus, S., D. W. Esplin, and D. M. Donaldson. 1954. Lack of bactericidal effect of mouse serum on a number of common microorganisms. *Science* 119: 877.

MIYA, MARCUS AND PHELPS

19. Marcus, S., F. Miya, L. J. Phelps, and L. W. Spencer. 1961a. Effect of acute and chronic low temperature stress on survival of mice challenged with Staphylococcus aureus. AAL TR 61-42, Project 8241-32, Arctic Aeromedical Laboratory, Fort Wainwright, Alaska.
20. Marcus, S., F. Miya, L. J. Phelps, and L. W. Spencer. 1961b. Studies on Klebsiella pneumoniae passed through mice maintained at low ambient temperatures. AAL TR 61-7, Project 8241-32, Arctic Aeromedical Laboratory, Fort Wainwright, Alaska.
21. Marcus, S., F. Miya, L. J. Phelps, and L. W. Spencer. 1962. Effect of low ambient temperatures on specific and nonspecific resistance. Seventh Quart. Prog. Rep. Contract AF 41(657)-311, Arctic Aeromedical Laboratory, Fort Wainwright, Alaska.
22. Mills, C. A., and L. H. Schmidt. 1942. Environmental temperatures and resistance to infection. *Am. J. Trop. Med.* 22:655-660.
23. Miraglia, G. J., and L. J. Berry. 1962. Secondary bacterial involvement following primary experimental infection in mice at 25° C and 5° C. *Bact. Proc.* p. 72.
24. Miya, F., S. Marcus, and E. H. Perkins. 1960. The properdin system in mice. *Proc. Soc. Exp. Biol. Med.* 105: 668-671.
25. Miya, F., S. Marcus, and E. H. Perkins. 1961. Cellular factors in resistance to acute bacterial infection. *J. Immunol.* 86:526-532.
26. Miya, F., L. Phelps, L. Spencer, and S. Marcus. 1962. Effects of exposure to low ambient temperatures on specific and non-specific resistance. *Fed. Proc.* 21: 278.
27. Muschenheim, C., D. R. Duerschner, J. D. Hardy, and A. M. Stoll. 1943. Hypothermia in experimental infections. III. The effect of hypothermia on resistance to experimental pneumococcus infection. *J. Infect. Dis.* 72: 187-196.

SPECIFIC AND NONSPECIFIC RESISTANCE

28. Pappenheimer, A. M., L. J. Kunz, and S. Richardson. 1951. Passage of Coxsackie virus (Connecticut-5 strain) in adult mice with production of pancreatic disease. *J. Exp. Med.* 94:45-64.
29. Pasteur, L., J. F. Joubert, and C. Chamberland. 1946. *Bull. Acad. Med.*, 2nd Ser., 7:432, 1878. From Topley and Wilson, *Principles of Bacteriology and Immunity*. Baltimore, Williams, and Wilkins, 3rd Edition.
30. Pillemer, L., L. Blum, I. H. Lepow, L. Wurz, and E. W. Todd. 1956. The properdin system and immunity. III. The zymosan assay of properdin. *J. Exp. Med.* 103: 1-13.
31. Previte, J. J., and L. J. Berry. 1962. Virulence and infection following acute exposure to cold. *Bact. Proc.* P. 72.
32. Rice, C. E., and C. N. Crowson. 1950. The interchangeability of the complement components of different animal species. II. In the hemolysis of sheep erythrocytes sensitized with rabbit amboceptor. *J. Immunol.* 65: 201-210.
33. Ross, O. A. 1956. The properdin system in relation to fatal bacteremia following total-body irradiation of laboratory animals. *Ann. N. Y. Acad. Sci.* 66: 274-279.
34. Sarracino, J. B., and M. H. Soule. 1941. Effect of heat, cold, fatigue, and alcohol on resistance of mice to human influenza virus. *Proc. Soc. Exp. Biol. Med.* 48: 183-186.
35. Schonbaum, E. 1960. Adrenocortical function in rats exposed to low environmental temperatures. *Fed. Proc.* 19: 85-88.
36. Selye, H. 1950. *Stress*. Acta, Inc., Montreal, Canada.
37. Smith, L. W., and T. Fay. 1939. Temperature factors in cancer and embryonal cell growth. *JAMA* 113: 653-660.
38. Sulkin, S.E. 1945. The effect of environmental temperature on experimental influenza in mice. *J. Immunol* 51: 291-300.

MIYA, MARCUS AND PHELPS

39. Sulkin, S. E., R. Allen, R. Sims, P. H. Krutzsch, and C. Kim. 1960. Studies on the pathogenesis of rabies in insectivorous bats. II. Influence of environmental temperature. *J. Exp. Med.* 112: 595-617.
40. Tannenbaum, A., and H. Silverstone. 1949. Effect of low environmental temperature, dinitrophenol or sodium fluoride on the formation of tumors in mice. *Cancer Res.* 9: 403-410.
41. Trapani, I. L. 1960. Cold exposure and the immune response. *Fed. Proc.* 19: 109-114.
42. Umbreit, W. W., R. H. Burris, and J. F. Stauffer, 1957. *Manometric Technique*. Minneapolis, Burgess Publishing Co., III Ed.
43. Wallace, W., H. M. Wallace, and C. A. Mills. 1942. Effect of climatic environment upon the genesis of subcutaneous tumors induced by methylcholanthrene and upon the growth of a transplantable sarcoma in C3H mice. *J. Nat. Cancer Inst.* 3: 99-110.
44. Wallace, W., H. Wallace, and C. A. Mills. 1944. Influence of environmental temperature upon the incidence and course of spontaneous tumors in C3H mice. *Cancer Res.* 4: 279-281.
45. Walker, D. C., and W. D. Boring. 1958. Factors influencing host-virus interactions. III. Further studies on the alteration of Coxsackie virus infection in adult mice by environmental temperature. *J. Immunol.* 80: 39-44.
46. Youngner, J. S. 1954. Monolayer tissue culture. I. Preparation and standardization of suspensions of trypsin-dispersed kidney cells. *Proc. Soc. Exp. Biol. Med.* 85: 202-205.

SPECIFIC AND NONSPECIFIC RESISTANCE

DISCUSSION

MIRAGLIA: You made a statement that the rectal temperature of the mouse equilibrates after 24 hours and then establishes a plateau that is 1°C higher than the temperature the animal started out with. We found the same thing in the first 24 hour period, but our plateau temperature has been approximately the normal temperature of the mouse, rather than 1°C higher. I was wondering if a possible explanation of this isn't the fact that animals, in our experience, anyway, lose their tails and ears after 14 to 21 days in the cold, and losing this cooling surface causes them to have a slight elevation in temperature. I noticed in Figure 9 that you kept your animals at 2°C for 30 days. Do you do this in all of your experiments?

MIYA: Our acclimatization period varies. At the present time, we are trying to establish some criterion for acclimatization and experiments are being conducted at the present time in which we are measuring the adrenal weight and animal weight. We intend to do eosinophil counts and we have even thought of checking for stress lymphocytes; I don't have any conclusive data yet, but I feel, based on what I have read in the literature, that if you used approximately thirty to forty or sixty days, this is considered in the opinion of most people to be an adequate acclimatization period.

MARCUS: How about the loss of tails and ears?

MIRAGLIA: Do your animals experience this? The animal loses the tail completely, in our experience, and I was wondering whether your animals do the same thing.

BERRY: Yours are grouped how?

MIYA: Five or ten together; at least five.

BERRY: With bedding?

MIYA, MARCUS AND PHELPS

MIYA: They have a slight amount of sawdust in the cage.

BERRY: This could make a difference.

MIYA: Our singly caged mice are placed in complete wire cages and do not have any bedding.

MIRAGLIA: Do these mice lose their tails?

MIYA: Occasionally you will see a singly caged mouse that will lose its tail completely.

PREVITE: Is your cage of a standard size, or intentionally smaller because you have only one mouse in it?

MIYA: The dimensions are approximately 4 in. by 4 in. by 7 in. per mouse.

PREVITE: He has plenty of room.

BERRY: After these animals have been acclimatized at low temperatures for forty-five days, have you subjected them to an even more acute exposure, say, -20° C, in a deepfreeze, for example. Can they survive a super stress longer than animals that have not been subjected to this long period of acclimatization?

MIYA: We haven't done any experiments like that.

BERRY: This might be of value, because altitude exposed mice that have "acclimatized" are able to withstand a hypoxic level for longer periods of time than animals that have not been at simulated altitudes. Possibly these animals that have been maintained at a low temperature, say of 2° C, for some time, may be able to withstand more acute cold stress. If you could show something of this type, I would be a little happier about calling them acclimatized to cold. Your results afford a very good argument for acclimatization, because animals with prior experience at low temperature respond to challenge differently from animals that have never had a previous experience. One

SPECIFIC AND NONSPECIFIC RESISTANCE

might be justified in calling them acclimatized, but are they acclimatized to cold? At least they have changed in such a way that their response to infection is different.

BLAIR: Did I understand you to say that the magnitude of reductions in oxygen uptake of the microorganisms at 32° C was about one-half of normal?

MIYA: One-half of that obtained at 37° C.

BLAIR: This is a rather striking bit of information. It really shakes me up a bit. We humans fancy ourselves as being the acme of biological development, and I don't know whether I am delighted or distressed to inform you that for the oxygen uptake in human beings at 32° C, the magnitude of reduction is exactly the same as it is with Staphylococcus aureus, so perhaps at least with regard to fundamental metabolism, we are not so different from our primitive brethren.

BERRY: Don't be surprised. I think this is a fundamental physiological fact. Some physiologists here would probably confirm that, wouldn't you, Dr. Vaughn? It is not surprising that a tissue subjected to any given temperature reduction would show approximately the same metabolic relationship.

MIRAGLIA: Have you found that the animals eat more when they are cold stressed?

MIYA: Yes, this was an inadvertant discovery. When we first started in this work, I didn't even know if the mouse could tolerate the cold, and on Friday, I put mice in the ice box and threw them a handful of food. On Monday, the mice were all uniformly dead, which I didn't quite understand. But this was the most reproducible thing. I noted that there was no food in the cage, and then I determined that they ate approximately five times that which they would normally eat at room temperature.

MIRAGLIA: I am very happy to hear this, because someone was wondering yesterday if cold was stress to the animal.

MIYA, MARCUS AND PHELPS

If food intake is any criterion, they certainly are stressed, because we have noticed the same thing.

SCHMIDT: In measuring the effect on conditioned organisms, you tested them after intraperitoneal injection at 32° C, yet rectal temperatures of your mice have been shown to be higher than those of the normal mouse after exposure to cold. I wonder why it is that you decided to investigate the characteristics of these organisms at a lower temperature? Why not investigate them at a higher temperature, because this is indeed what you have found in the experimental system.

MIYA: This was done when we first started into this work. We briefly scanned the literature and most of the reports indicated that there was a possible drop in rectal temperatures. We didn't have any suitable temperature measuring equipment at that time, so we decided to approach the problem from a back door; to find out what these organisms would do at different temperatures, anticipating this drop. However, we found quite to the contrary that the temperature elevated.

SCHMIDT: You haven't had the occasion yet to investigate the growth curves or other characteristics of these organisms at one or two degrees above the normal rectal temperature?

MIYA: No, we haven't.

REINHARD: In the experiments on the isolates of the "conditioned bacteria", how many isolates did you use from each group of mice for determination of oxygen uptake?

MIYA: These were pooled isolates.

REINHARD: How did you pool them?

MIYA: We killed the animals and then opened up the peritoneal cavities aseptically and just removed the exudate.

NUNGESTER: Without plating?

SPECIFIC AND NONSPECIFIC RESISTANCE

MIYA: Yes, we removed the peritoneal exudate, pooled the samples, and reinjected this material into another group of animals.

MARCUS: And the data shown from the isolates would be organisms from the seventh pool.

REINHARD: How many determinations did you make of the oxygen requirements; how many parallel experiments did you run?

MIYA: Each Warburg flask was set up in duplicate, and the runs were done three times.

REINHARD: Isn't it possible you could have run into chance variations? I would be more comfortable if you said you ran fifty determinations.

MIYA: I could have shown a series of curves that would essentially show what I have shown here. I chose, rather than to confuse the issue, to show a group of typical curves obtained for any one determination.

REINHARD: Then how did you test these organisms after they had grown out again to see whether they recaptured their original state or whether they remained the same in their oxygen requirements?

MIYA: I don't think I quite understand.

NUNGESTER: He means, if they're carried along as a pure line culture, how long do they retain this altered characteristic?

MIYA: After the seventh passage, we did the oxygen uptake studies and we didn't do any more after that. We merely saved those organisms for the challenge in subsequent experiments.

WALKER: I'd just like to comment on this curious, interesting difference in the effect of cold on various strains of mice that Dr. Miraglia mentioned. In our strain of Wisconsin mice,

MIYA, MARCUS AND PHELPS

the body temperature is very easily changed, and this isn't a matter of how far you insert the probe into the rectum. We use one centimeter. We would go further, but we get a higher incidence of perforated colon and so on. This difference among mouse strains is fairly important, it seems to me, because I am prepared to propose that in virus infections, the change in body temperature is quite important, and I am really more impressed with the effect of the temperature of the tissues than with factors like stress. The effect of cold on viral infections has been quite variable, quite contradictory, and I wonder if the differences among not only species, but among strains of mice may not be partly responsible for this variability. As Dr. Campbell indicated, and as my experience has been, too, you can hardly change the body temperature of a rabbit, regardless of what you do. If you do go down low enough in environmental temperature, the temperature suddenly begins to change and it goes right on down and the animal quickly dies; but up to that point, you cannot really lower it. But in our experience, the mouse temperature is quite readily changed. If you raise the environmental temperature, you raise his body temperature.

MIYA: If you plot distance into the rectum on the abscissa and the temperature on the ordinate, you notice almost a linear relationship.

MARCUS: From two centimeters down to one?

MIYA: Well, we took it at .5, 1.0, 1.5, and 2.0 cm down.

PREVITE: I think this has also been confirmed by Halberg and Spink.¹ In my own experiences, the distance the probe was inserted made a significant difference in the rectal temperature recorded.

MARCUS: We have had no difficulty with rupturing. We haven't penetrated too many.

¹ Halberg, F., and W. W. Spink. 1956. Laboratory Investigation.

SPECIFIC AND NONSPECIFIC RESISTANCE

PREVITE: Halberg and Spink advise 23 ± 2 mm.

MIYA: Our mice here have been acutely exposed to cold with the rectal probe at 2 cm depth, and the temperature remains at this level, but at 1 cm depth, it falls. This is quite similar to the type of curves that you obtained.

WALKER: I have never measured body temperature over a brief period of time like that. I have always tested the amount of time it takes for the probe to come to a steady state, but I have not determined the temperature in the first few hours after exposure.

BLAIR: One final comment, if I may, about the accuracy of temperature measurement. This problem has been kicked around, of course, in large animals, and it has been demonstrated that the variations seem to occur during the dynamic changes in temperature, but once the temperature is stabilized, it really doesn't matter where you measure the so-called core temperature. I can't speak for the smaller animal, but certainly in the large animals it does make a difference of what the point of interest is. The changes occur physiologically and physically during the actual changes of temperature, but once the temperature is stabilized, it doesn't really matter where it's measured.

WALKER: Benzinger finds considerable difference between rectum and esophagus temperatures, as I recall, in the first few hours, but after that, it makes no difference.

BLAIR: So it depends upon what you are interested in studying.

WALKER: I am not quite clear as to whether or not the effect of cold on Cocksackie virus infection that you described required continued exposure to cold to get this increased mortality.

MARCUS: We didn't carry out any experiments that discontinued; in other words, the animals were put in the cold and they stayed to the continuation of the experiment, but there was no discontinuous effect as you studied.

MIYA, MARCUS AND PHELPS

WALKER: I was wondering about the difference between your mice and mine in readjusting their temperatures. That emphasizes the difference in mice, I believe.

MARCUS: Well, actually we are both using albino Mus musculus.

WALKER: Yes, but we are using a colony that we have that is originally derived from Webster-Swiss mice.

PREVITE: Are your housing conditions the same?

WALKER: Ours were in wire cages with metal bottoms and no bedding, but with five or six animals per cage.

PREVITE: No bedding? I think you mentioned using sawdust and something else. I had a question about rectal temperatures in acclimatized mice. It rather intrigues me in that you report the rectal temperatures are 1°C to 2°C higher 40 to 45 days after acclimatization.

MARCUS: No, not 45 days. This occurred within 24 hours. Dr. Miya showed a chart of the effect of measuring their temperatures as a function of the distance in the colon from a depth of 2 cm. He had points of 2.0, 1.5, 1.0, and .5 cm in the animal.

PREVITE: Does this persist, this elevated temperature, or is it something that is transient?

MARCUS: No, it persists and remains fairly steady.

PREVITE: This is what I mean. Once this temperature goes up, it stays up.

MARCUS: That is true, 1°C to 2°C higher than the normal, and this is with the probe uniformly inserted all the time.

PREVITE: Do you have any possible explanation? Why should the temperature go up? I have no idea why it would go up.

MIYA: As you probably know, there is a report, which I be-

SPECIFIC AND NONSPECIFIC RESISTANCE

lieve Herrington² wrote which showed that the metabolic rates of mice increase linearly as the ambient temperature decreases. We feel that under these conditions, the "thermostat" has now been set at a new higher level in an attempt at maintenance of homeostatic conditions.

PREVITE: This animal, being a homeotherm, would maintain a constant body temperature regardless of the environmental temperature as long as it was able to.

TRAPANI: I am curious about one thing: Is the life span of the mouse, or any other animal for that matter, the same under these conditions of cold exposure as compared to the room temperatures? Our time relationships should be weighed if the life span is different.

MARCUS: I don't know what the life span of the mouse is if you put him in the cold.

PREVITE: Barnett and his group³ have done work on this. They report that mice may be successfully reared in the cold. However, mice reared in the cold survive longer without nesting material than those transferred from a warm room and also deprived of cold. Selye⁴ has shown extensive damage to critical organs can result from exposure of rats to cold.

MARCUS: Well, with these experiments that have gone up to three months involving animals in cold, you have discussed some of the modest changes that occur, at least they seem modest to me, in the periphery of the animal, and I am not aware of any gross changes that occur, any gross pathology that occurs in the animals that are autopsied which could be ascribed to cold alone. With regard to what you said, though, a moment ago, about the mouse being a homeothermic animal and you would expect it to maintain its temperature once it is stabilized at a fairly con-

² Am. J. Physiol. 129: 123. 1940.

³ Barnett et al. 1959. Quart. J. Exp. Physiol. XLIV.

⁴ Selye, H. 1943. Rev. Canad. Biol. 2.

MIYA, MARCUS AND PHELPS

stant level: now, I think this has been the experience that you have had, too.

PREVITE: My experiments were much shorter; they were carried out within a day or two.

MARCUS: Now, just a second. You said in a day or two. What happened in a day or two?

PREVITE: My results would agree in general with those that you presented for the first day. The only thing I am puzzled about is why, after it does stabilize, does it stabilize itself at a temperature 2° C or 3° C higher?

MARCUS: It is easy to guess about this. The animal in the cold is passing off a lot of heat, and it has to maintain its temperature. It eats five times or more as much as an animal does at room temperature. We never measured the metabolic rate involved, but it may be that it just sets this whole mechanism up.

VIERECK: Maybe this would explain some of the contradictory results. If you put an animal in the cold, it is good and bad for him at the same time. It is bad for him if it is a stress; consequently, if the animal is stressed by cold, he is suffering, and thus less well able to cope with other stresses such as infection; and at the same time, cold exposure could be considered as being good for the animal inasmuch as it does speed up metabolism. He is eating more, his oxygen consumption is higher, et cetera. Now, here is an idea: Maybe this general speeding up of metabolism includes protein metabolism. I don't know of any evidence for this one way or another, other than food intake studies. Now, if protein metabolism is speeded up, is it reasonable to suspect that antibody formation would be coupled with this and the synthesis of antibodies would be automatically in a higher gear after the animal has been in the cold? What do you think of that?

MARCUS: I discussed this with Dr. Trapani and he pointed out that Dr. Whipple, employing plasmaphoresis, showed that there was no protein that was as metabolically effective as gamma

SPECIFIC AND NONSPECIFIC RESISTANCE

globulin for use as a metabolic thing. The point that I am trying to make is that antibody, as such, is not only a constituent of gamma globulin, but it is a part of a protein which can be employed by the animal as a source of amino acid for protein metabolism very nicely; and I am sure that under these circumstances, the turnover rate of protein in the animal is related to antibody production; at least I feel there must be a correlation here; however, I am certainly not an authority in that area.

CAMPBELL: We have shown that the half-life of protein decreased rather measurably under cold. There has been some preliminary work done which has been extremely interesting with tagged amino acids and tagged gamma globulin, and it would seem, as far as antibody protein goes, that in the normal synthesis, the body prefers amino acids to gamma globulin. When you start immunizing or bleeding, the body seems to prefer gamma globulin. So if you label these two things, in one situation, the amino acids will be incorporated; in the other case, the gamma globulin is broken down and reincorporated.

McCLAUGHRY: In relation to Dr. Preville's question about homeothermia, I don't believe the temperature of a homeothermic animal is anywhere near as fixed as has been discussed here. As a matter of fact, Adolf⁵ showed that if the conditions vary the thermostat may be re-set at a slightly different level, perhaps 1° C or 2° C from the original, after acclimatization. This varies in different physiological regulatory mechanisms, and I think that this has to be taken into account in the experiment that has been reported.

BERRY: Dr. Miraglia has some results related to this point. He was given some black mice from NIH and DBA, weren't they?

MARCUS: They have a variety of types; DBA, CBA.

BERRY: These mice were placed at 5° C. They were all dead within 24 hours. They were completely unable to withstand this

⁵ Am. J. Physiol. 166: 62. 1951.

MIYA, MARCUS AND PHELPS

temperature. We were trying to compare mice of a different genetic makeup, but were defeated in the attempt.

MARCUS: Didn't we have a similar experience with some dark mice?

MIYA: We have not used black mice in any of our experiments.

BERRY: This shows the same difference in mice in a very dramatic way. The other question I have is in regard to this endotoxin. You injected it 48 hours before the infectious challenge and within this period of time, as I recall, in some cases there should be an increase in non-specific resistance capable of protecting them against bacterial challenge.

MARCUS: We chose that time because it has been subscribed to, and we had the same results. There is a very critical time for inducing protection. You remember that this time is quite critical, because if you challenge about four hours after endotoxin administration there is a decrease in resistance. Also, if you wait much beyond three days, it is all gone.

BERRY: Has an increase in non-specific resistance ever been shown to protect against a viral challenge under any condition?

MARCUS: Not that I know of.⁶

BERRY: I don't remember either.

TRAPANI: Does anyone ever use AKR mice for long term experiments on cold exposure? In regard to some of the remarks you made about the neoplasms and virus studies, it would be interesting to study the AKR mouse. This strain of mouse shows spontaneous development of leukemia and lymphoma at about nine months to one year of age, and this condition has been considered to have a viral etiology. It would be interesting to use the AKR

⁶ See article by Nemes and Hilleman, Proc. Soc. Exp. Biol. Med. 110: 500, 1962, in which endotoxin is shown to increase resistance in mice against some but not all virus infections.

SPECIFIC AND NONSPECIFIC RESISTANCE

mouse; just put them into the cold and see what happens.

METCALF: I would like to make just one comment. I think we are all aware of the importance of the genetics of the host that we are using, but I wonder if there might not be more subtle relationships that we may be missing. For example, Weir⁷ and others have utilized sub-lines within a given species which vary significantly with regard to blood pH. This may be of importance in phagocytosis and perhaps some of the mechanisms that you are measuring. I wonder if we may be overlooking these relationships and consequently failing to assess or evaluate properly some of the results obtained.

SULKIN: My ears perked up at the comment you made, and I happened to think of the experiments that we recorded a few years ago on bacteriophage clearance in people and animals -- rabbits, in this case. As you know, when bacteriophage has been introduced intravenously into a rabbit it is inactivated quite promptly and you can't detect phage in an animal, actually, within a couple of hours. And we have the impression that perhaps properdin was the component that was inactivated and undertook an experiment in which we treated these animals with zymosan, and zymosan did something that depressed properdin, because phage would then persist, and this has a bearing on the point that Dr. Berry mentioned.

MITCHELL: My one question is this: Dr. Berry and his group and the group at Wisconsin, got to comparing notes. We have experiments wherein one is performed at pressures of about 100 mm less atmosphere than are the ones that are performed at Bryn Mawr or at Wisconsin. I wonder if this small difference may make them into different animals. The reason I am saying this is because I knew of your own experience with altitude exposures, and I don't know whether you combined this with cold.

BERRY: I would like to say that Joe Wilson and I have been

⁷ Weir, J. A. 1949. J. Infect. Dis. 84: 252-274.

MIYA, MARCUS AND PHELPS

exchanging mice between Bryn Mawr and Madison because we have not been able to get duplicate results, and they're interesting to both of us. As a matter of fact, when we ship these mice by air express, the mice have been changed. The Madison mice shipped to Bryn Mawr are very different animals.

MITCHELL: Well, it will be interesting when you gentlemen eliminate this additional factor of 100 mm of mercury and see what happens.

VIRULENCE AS A FACTOR IN HOST RESPONSE TO BACTERIAL INFECTION AT LOW ENVIRONMENTAL TEMPERATURE^{1,2}

Joseph J. Previte³ and L. Joe Berry

Department of Biology, Villanova University
Villanova, Pennsylvania

Department of Biology, Bryn Mawr College
Bryn Mawr, Pennsylvania

ABSTRACT

The purpose of this report has been to investigate the effect of acute exposure to cold on the response of mice to *Salmonella typhimurium* and *Staphylococcus aureus*, and to injections of lipopolysaccharides derived from Gram negative organisms. Mice maintained in individual compartments without bedding following infection with an avirulent strain of either *Salmonella typhimurium* or *Staphylococcus aureus* are more susceptible when exposed continuously to 5° C than they are when exposed to 15° C or to 25° C. These differences are not observed when virulent strains are used, while acclimatization to cold for two weeks fails to alter the response to the avirulent organisms. Mice kept at 5° C post-injection are sensitized 250-fold to pasteurized *Salmonella typhimurium*, and about 10-fold to lipopolysaccharide derived from *Serratia marcescens* compared to control animals housed at 25° C. Mice given an LD₇₅ dose of lipopolysaccharide and placed at 5° C for 12 hours before transfer to 25° C are as susceptible to the endotoxin as mice kept continuously in the cold. Conversely, mice given the same dose and retained at 25° C for 6 or 12 hours before placing them at 5° C are almost as resistant as mice kept continuously at 25° C. The period of sensitization to lipopolysaccharide following cold exposure was paralleled by the time at which a drop in body temperature occurred following the low temperature stress and/or endotoxin poisoning. Protection was afforded the cold exposed mice against endotoxin poisoning by exogenously administered cortisone acetate while 8 units of ACTH enhanced the lethal effects of the toxin. The adrenal response of the host to temperature stress seems to be of paramount significance in determining sensitization to lipopolysaccharide.

¹ Some of the data presented in this paper has been published in the *Journal of Infectious Diseases* 110: 201-209, 1962.

² This work was supported in part by contract AF 41 (657)-340 between Bryn Mawr College and the Arctic Aeromedical Laboratory.

³ Present Address: Zoology Department, Smith College, Northampton, Massachusetts.

PREVITE AND BERRY

Many individuals assume that low environmental temperature plays a role in predisposition to infectious disease. While there is a relative dearth of scientific evidence to support this concept, a few reports have appeared in the literature. Some indicate that cold increases susceptibility to specific infections while others demonstrate an increase in survival following low temperature exposure (Girone, 1962). It seemed of import, therefore, to shed further light on this complex problem. The results described below demonstrate the response of cold exposed mice to infection with Salmonella typhimurium, Staphylococcus aureus, and to injection of endotoxins derived from Gram negative bacteria.

MATERIALS AND METHODS

During the experimental period, 21 ± 2 gm Carworth farm, CF-1 female mice were housed singly and maintained in plexiglass compartments without bedding. Mice could thus be isolated one from one another so that a group of ten animals could be housed individually in a total area measuring 8 in. x 10 1/2 in. The importance of single housing (Kulka, 1961) and lack of nesting material (Barnett et al., 1959) as stress factors in cold studies have been demonstrated. Single housing eliminates the huddling of mice and conservation of body heat that normally accompanies this activity. Lack of nesting material prevents burrowing and thereby deprives the animal of insulatory material available under usual caging conditions, thus accentuating heat loss. The cold exposed animals were maintained in walk-in refrigerators at $5 \pm 1^{\circ}$ C or $15 \pm 1^{\circ}$ C. Room temperature controls were kept in an air-conditioned laboratory at $25 \pm 2^{\circ}$ C. The animals were given food (Dietrich and Gambrill's pathogen-free mouse biscuits) and water ad libitum. For the single experiment involving high temperature exposure, the mice were placed in compartments held in an incubator with a controlled temperature of $35 \pm 2^{\circ}$ C. Three liters of air pre-warmed in a water bath were passed through the incubator each minute. The rate of air flow was determined by a flow meter (Fisher).

VIRULENCE AND BACTERIAL INFECTION

Injections of bacteria derived from 17 hour brain-heart infusion cultures, and lipopolysaccharide derived from Serratia marcescens (Difco), as well as heat-killed Salmonella typhimurium, were suspended in 0.5 ml of non-pyrogenic saline (Baxter's) and administered at the start of each experiment. All injections were given intraperitoneally other than staphylococci which were given intravenously via the tail vein of the mouse. A viable count of 10^9 cells per ml based on numerous experimental determinations was assumed for the undiluted cultures. Staphylococcal toxins were obtained as sterile filtrates of the contents of diffusion chambers which were constructed with Viscosedialyzing membranes. Staphylococcus aureus, Giorgio, had been grown inside the chambers for 10 to 14 days after implantation in the peritoneal cavities of mice. This technique is described in detail by Houser and Berry (1961). Mice were injected intravenously with 0.1 ml of a 1:32 dilution of the filtrate and placed immediately at 5° C or 25° C. The material was supplied by Mr. Enoch D. Houser.

Rectal temperatures were determined by inserting a thermistor probe approximately 21 mm into the rectum of mice for twenty seconds. The temperature was read from a telethermometer (Yellow Springs Instrument Company, Yellow Springs, Ohio) to which the probe was connected. A mercury thermometer was used to calibrate the instrument.

Five mg of cortisone was administered subcutaneously as a suspension of cortisone acetate (Nutritional Biochemicals, Cleveland) in 0.5 ml of non-pyrogenic isotonic saline solution. The suspension, stabilized with a drop of Tween 80 detergent was prepared in a glass homogenizer with teflon pestle and used immediately thereafter. Adrenocorticotrophic hormone, ACTH, (Armour Laboratories, Chicago) was injected as a gelatin suspension (Acthar Gel) containing two units per 0.05 ml.

The significance of differences in survival due to experimental treatments was determined by the chi-square test using Yate's corrected formula (Croxtton, 1959) and that for rectal temperature measurements was determined by the rank order test (White, 1952). For some treatments mean survival times were calculated, including only the mice that died. LD₅₀ dosages were determined

PREVITE AND BERRY

Days Post Infection	Number of survivors at		
	5° C	15° C	25° C
0	42	32	41
2	39	32	41
4	32	30	40
6	19	26	36
8	9	20	27
10	3	17	22
12	1	8	19
14	0	8	16

Table 1. Survival of mice infected with 10^5 *Salmonella typhimurium*, SR-11-A. (Derived from data presented in J. Infect. Dis. 110: 201-209, 1962.)

according to the method of Reed and Muench (1938).

RESULTS

Infection with S. Typhimurium, SR-11-A

Mice were infected intraperitoneally with 10^5 viable cells of *S. typhimurium*, SR-11-A. This strain was derived by chance from an agar slant culture of SR-11 which had been stored at 5° C for a period of several months. On transfer to brain-heart infusion broth and injection into mice, it was found to have lost much of its virulence. The animals were divided into three groups and placed at temperatures of 5° C, 15° C, and 25° C immediately after infection. The pooled results are presented in Table I. None of 42 mice survived at 5° C while 25 per cent (8 of 32) of those at 15° C and 39 per

VIRULENCE AND BACTERIAL INFECTION

Days Post Infection	Number of Survivors at		
	5° C	15° C	25° C
0	32	32	31
2	31	32	30
4	25	24	20
6	5	4	6
8	1	0	3
10	0	0	3
12	0	0	3
14	0	0	3

Table II. Survival of mice infected with 10^5 *Salmonella typhimurium*, SR-II. (Derived from data presented in J. Infect. Dis. 110: 201-209, 1962)

cent (16 or 41) at 25° C were alive at 14 days. Deaths resulted sooner at 5° C than at the two higher temperatures ($P < 0.005$ in both cases) and mean survival times in animals succumbing to infection were 6.0, 8.5, and 8.7 days in order of increasing temperature. The experiments were terminated at 14 days in order to avoid ambiguities in interpreting the results. Five degree exposure alone can cause deaths if mice of the strain used are maintained at this temperature for periods longer than two weeks.

In a single experiment, 12 mice that had survived acclimatization for two weeks at 5° C were then infected with 10^5 SR-IIA. Fourteen days later only one of twelve was still alive. Thus, survival at 5° C in mice with this infection does not differ with or without two weeks of prior acclimatization ($P = 0.3$).

Infection with *S. Typhimurium*, SR-II

Mice were infected intraperitoneally with 10^5 cells of virulent

PREVITE AND BERRY

Days Post Infection	5 x 10 ⁶ cells			Infectious Dose 5 x 10 ⁴ cells			2 x 10 ⁴ cells		
	5°	15°	25°	5°	15°	25°	5°	15°	25°
0	10	10	10	12	10	10	33	10	32
2	6	6	4	11	10	10	33	9	32
4	0	0	0	11	10	10	32	9	31
6				1	4	6	19	3	22
7				1	3	5	6	2	16
8				0	1	3	3	2	15
10					0	1	1	0	6
12									5
14									4

Table III. Survival of mice infected with *Salmonella typhimurium*, SR-11, and exposed to 5° C, 15° C, and 25° C. (Derived from data presented in J. Infect. Dis. 110: 201-209, 1962.)

S. typhimurium, strain SR-11. The animals were divided into three groups and placed without delay at 5° C, 15° C, and 25° C. The results in Table II demonstrate no significant difference in survivorship and the mean survival times were, in order of increasing temperature, 5.4, 5.3, and 4.7 days. Cold was thus without influence either on survivorship or survival time of mice infected with this particular dose and strain of *Salmonella*.

The effect of graded doses of SR-11 was then evaluated in order to determine whether an effect of temperature on the infectious process may have been masked by an overwhelming infection. Mice were infected with seven different doses of bacteria, ranging from 500 to 5 million cells and then placed at 5° C, 15° C, and 25° C. The animals were more successful, temporarily, in resisting the lethal effects of the virulent salmonellae when housed at room temperature (Table III). At seven days after infection with 20,000 SR-11 only 18.2 per cent (6 of 33) of the mice survived at 5° C, whereas 50 per

VIRULENCE AND BACTERIAL INFECTION

Days Post Infection	Number of Survivors at		
	5° C	15° C	25° C
0	22	22	24
2	15	22	22
4	12	18	21
6	11	14	20
8	10	11	19
10	10	10	19
12	2	8	19
14	2	8	19

Table IV. Survival of mice infected with 10^8 *Staphylococcus aureus*.
(Derived from data presented in J. Infect. Dis. 110: 201-209, 1962.)

cent (16 of 32) survived at 25° C ($P < 0.02$). This difference was abolished by the fourteenth day. With infectious doses as low as 500 cells or as high as 5 million cells, both survival time and survivorship of mice infected with SR-11 were largely independent of the environmental temperature at which the post-infection period was spent.

Infection with *Staphylococcus*

Mice were infected intravenously with 10^8 cells of a relatively avirulent variant culture of *Staphylococcus aureus*, Giorgio. They were placed immediately after infection at 5° C, 15° C, or 25° C. After 14 days, 9 per cent (2 of 22) survived at 5° C, 36 per cent (8 of 22) survived at 15° C, and 79 per cent (19 of 24) survived at 25° C. Thus with this particular organism, survival was decreased significantly by 5° C ($P = 0.001$) or 15° C exposure ($P = 0.01$) when compared to that at 25° C (Table IV).

Mice injected intravenously with 10^8 virulent *Staphylococci* were about equally susceptible both as to survivorship and survival time

PREVITE AND BERRY

5° C and 25° C. Three of ten animals survived at each temperature, and the mean survival times were 2.9 days at 5° C and 3.6 days at 25° C.

With a smaller infectious dose of virulent cells (500 bacteria), four of eight mice survived at 5° C while six of eight were alive at 25° C within 14 days of the initial infection ($P > 0.05$). Once again as with salmonellosis, the more virulent organism seems to produce a fatal infection with little influence from the environmental temperatures at which the animals were housed.

Injection of Staphylococcal Toxin

A total of 16 mice were injected intravenously with 0.1 ml of a 1:32 dilution of a staphylococcal toxin derived as a sterile filtrate from a viscose diffusion chamber in which the bacteria had been grown as described previously. Ten were placed at 5° C and the remainder were held at 25° C. Three died at 5° C and none at 25° C. For the number of animals involved this is not significant. However, if all mice previously given this amount of toxin (by Mr. E. D. Houser) at room temperature are added to the 25° C group (a total in excess of 30, none of which died), it would suggest that cold sensitizes mice to Staphylococcal toxins. The limited supply of toxin prevented a more extensive test.

Heat-killed *S. Typhimurium*

Groups of 10 mice were given graded numbers of pasteurized *S. typhimurium* intraperitoneally. The LD₅₀ dose at 5° C was 8×10^6 cells while at 25° C, it was 2×10^9 cells. Thus, cold exposure increases 250-fold the lethal effects of heat-killed salmonellae.

Two groups of mice were then acclimatized to 5° C and 25° C for 28 days. At the end of this period, 1.6×10^8 heat-killed cells were injected, and both groups of mice were placed at 5° C. Nine of ten cold acclimatized mice survived, while only two of ten non-cold acclimatized animals lived ($P = 0.008$). However, 20 per cent or more of the original group of mice at 5° C usually die during a

VIRULENCE AND BACTERIAL INFECTION

Days Post Injection	Controls 5° C	Treatment and Survivors		
		25° C	12 Hrs. 5° C	21 Hrs. 5° C
0	40	10	30	10
1	9	10	14	0
2	9	10	14	0
Per cent Survival	22.5	100.0	46.7	0

Table V a. Sensitization of Lipopolysaccharide in the cold. All mice received 60-65 μ g of Endotoxin. (Derived from data presented in J. Infect. Dis. 110: 201-209, 1962.)

Days Post Injection	Controls 5° C	Treatment and Survivors		
		25° C	6 Hrs. 25° C	12 Hrs. 25° C
0	40	10	30	15
1	9	10	22	15
2	9	10	21	15
Per cent Survival	22.5	100.0	70.0	100.0

Table V b. Protection against Lipopolysaccharide by 25° C exposure. All mice received 60-65 μ g of Endotoxin. (Derived from data presented in J. Infect. Dis. 110: 201-209, 1962.)

PREVITE AND BERRY

month long acclimatization. Therefore, it is not completely clear whether acclimatization to cold actually enhances the resistance of the mice to endotoxin or merely serves to select more resistant animals for the study.

Injection of *Serratia Marcescens* Lipopolysaccharide

The LD₅₀ doses of *S. marcescens* lipopolysaccharide (Difco) were determined to be 38.6 μ g, 408 μ g, and 162.5 μ g at ambient temperatures of 5° C, 25° C, and 35° C respectively. Therefore, both low and high environmental temperature sensitize mice to lipopolysaccharide.

Timing the Effects of Endotoxin at Different Temperatures

Mice were given 60-65 μ g of endotoxin (*S. marcescens* lipopolysaccharide) placed immediately at 5° C, and at intervals thereafter returned to room temperature. Exposure to cold for 12 hours (46.7 per cent survival) sensitizes mice to bacterial lipopolysaccharide almost as completely as continuous exposure (22.5 per cent survival) ($P > 0.05$, Table Va). Survival of 25° C controls (100 per cent) is significantly different from that of 5° C controls (22.5 per cent, $P < 0.001$). It is evident that the response of cold exposed mice to this dose of endotoxin is determined to a great extent within a 12 hour period. Experiments with heat-killed salmonellae (1.6×10^8 cells, which is more than an LD₅₀ dose), or with smaller doses of lipopolysaccharide (40 μ g) produced similar results.

Mice were then given 60-65 μ g of lipopolysaccharide, retained at room temperature, and at intervals thereafter placed in the 5° C room. Survivorship in the groups maintained at 25° C for six hours (70 per cent) and twelve hours (100 per cent) differed significantly from that of mice continuously exposed to 5° C after endotoxin injection (22.5 per cent, $P < 0.001$ in both cases; Table Vb). With a lower dose of lipopolysaccharide (40 μ g) similar results were obtained. Thus the mouse at normal room temperature (25° C) is able to render non-lethal within a 6-12 hour period a dose of endotoxin that is about 75 per cent lethal at an ambient temperature of 5° C.

VIRULENCE AND BACTERIAL INFECTION

Time Post Injection	Treatment and Temperatures		
	25° C Controls	5° C Controls	25° C & 408ug LPS 5° C & 50ug LPS
0 Hrs. (9:15 a.m.)	37.4 ± 0.4 (10)		
6 Hrs.	37.5 ± 0.5 (10)	36.1 ± 0.5 (10)	36.4 ± 0.9 (10) 29.6 ± 3.5 (20)
12 Hrs.	37.9 ± 0.6 (10)	36.3 ± 0.5 (10)	34.8 ± 1.7 (10) 29.7 ± 4.6* (9)
24 Hrs.	36.6 ± 0.8 (10)	36.2 ± 0.4 (10)	

Table VI. Average rectal temperatures of Lipopolysaccharide poisoned mice. *The rectal temperature of moribund mice was frequently below the lower limits of the thermometer (20° C). In these cases the temperature was recorded as 20° C in order to compute the arithmetic mean for the number of animals listed in parentheses. Each value represents the mean plus or minus the standard deviation from the mean of the number of animals studied.

PREVITE AND BERRY

Rectal Temperatures

Endotoxin has been reported to elicit hypothermia in mice (Halberg and Spink, 1956; Berry et al., 1959). It seemed appropriate to determine whether or not the onset and duration of hypothermia coincided with the period during which the mice were sensitized to this poison by cold exposure. The rectal temperatures of mice maintained at 25° C versus those at 5° C differed significantly ($P < 0.001$) after six (37.5° C vs. 36.1° C respectively) and 12 hours of exposure (37.9° C vs. 36.3° C). These differences disappeared within 24 hours at the two different ambient temperatures ($P > 0.05$) (Table VI).

The rectal temperature of 25° C controls (37.5° C) was significantly different ($P < 0.05$) from that recorded in mice that had received an LD₅₀ dose of lipopolysaccharide six hours previously (36.4° C). Within twelve hours the difference was even more marked between these two groups (37.9° C vs. 34.8° C respectively, $P < 0.001$). The rectal temperature of 5° C control mice and that of mice given an LD₅₀ dose of lipopolysaccharide prior to exposure to this same temperature differed markedly at 6 hours (36.1° C vs. 29.6° C) and at 12 hours (36.3° C vs. 29.7° C, $P < 0.001$ in both cases).

Thus hypothermia is evident within 6-12 hours in 5° C controls as well as lipopolysaccharide-poisoned animals exposed to 25° C. It is most severe in lipopolysaccharide-poisoned animals maintained at 5° C post-injection.

Hormone Administration and Survivorship

Cortisone acetate. It has been reported that adrenalectomized rats are killed by one-thousandth the dose of endotoxin required to kill normal animals (Brooke et al., 1959). The report that the administration of exogenous corticoids protects endotoxin poisoned animals was described as early as 1951 by Duffy and Morgan and has been repeatedly confirmed since then (Berry et al., 1959). However, whether the same protective effect would be evident in animals whose metabolism was elevated by cold exposure remained to be determined.

VIRULENCE AND BACTERIAL INFECTION

Days Post Injection	50ug LPS	Corti- sone & 50ug LPS	70ug LPS	Corti- sone & 70ug LPS	Cortisone Controls
0	30	26	40	30	10
1	19	23	0	24	10
2	19	23	0	24	10
Per cent Survival	63.3	88.5	0	80.0	100.0

Table VII. Hormonal protection against Lipopolysaccharide. Treatment and survivors.

With this in mind, five mg of cortisone acetate was administered subcutaneously immediately before intraperitoneal injection of 50 μ g of lipopolysaccharide (endotoxin). When the animals were maintained at 5° C post-injection survivorship was slightly but not significantly greater ($P>0.05$) in hormone treated mice than in animals not receiving hormone (88.5 per cent vs. 63.3 per cent, Table VII). However, with a larger quantity of lipopolysaccharide, the protection afforded by cortisone was clearly demonstrated. When an LD₁₀₀ dose (70 μ g) of lipopolysaccharide was given to mice exposed to 5° C after injection, all 40 controls died, while 24 of 30 mice given 5 mg of cortisone acetate survived ($P<0.001$). Cortisone alone killed no animals.

Adrenocorticotrophic Hormone

Exogenous ACTH administration has been reported to sensitize mice to the lethal effects of endotoxin. This occurs presumably because glyccorticoids are released too promptly in animals that receive both endotoxin and ACTH (Berry and Smythe, 1959; 1961) to fulfill the protective action the corticoids normally afford against endotoxin poisoning alone. The experiments described below were carried out to determine whether or not cold exposed animals would be further sensitized to lipopolysaccharide if given ACTH. The re-

PREVITE AND BERRY

Days Post Infection	50ug LPS	2 Units ACTH & 50ug LPS	8 Units ACTH & 50ug LPS	8 Units ACTH
0	30	30	30	14
1	19	15	3	14
2	19	15	3	14
Per cent Survival	63.3	50.0	10.0	100.0

Table VIII. Sensitization to Lipopolysaccharide by ACTH. Treatment and survivors.

sults indicate that this does occur (Table VIII). Two or eight units of ACTH were administered subcutaneously immediately before 50 μ g of lipopolysaccharide and subsequent exposure to 5° C. Survival in controls (63.3 per cent) was slightly but not significantly higher ($P>0.05$) than that of animals receiving 2 units of ACTH (50 per cent), but was significantly higher ($P<0.001$) than that in animals receiving 8 units of this hormone (10 per cent survival). Eight units of ACTH alone did not kill any of 14 mice.

DISCUSSION

Following experimental infection with *S. typhimurium*, the greater susceptibility of cold exposed mice (5° C, measured in terms of both survivorship and survival time) compared to those maintained at usual room temperature (25° C) is evident only when a comparatively avirulent strain is used (SR-11-A). Thus, a situation in which the outcome of a host-parasite interaction is intimately linked to the genetic constitution of the microorganism involved is evident. This may be related to the findings of Schneider (1949) and Schneider and Zinder (1956). They showed that diet altered the out-

VIRULENCE AND BACTERIAL INFECTION

come of salmonellosis only when a mixed population of virulent and avirulent pathogens was inoculated into the mice. As an alternative possible explanation, the results of Muschenheim et al., (1943) should be mentioned. They found that in rabbits infected with a virulent Type I strain of pneumococcus the only effect of reduced body temperature was a decrease in the local inflammatory response. However, with a relatively avirulent Type III strain, overwhelming bacteremia and death resulted as well. These investigators concluded that reduction in body temperature can alter resistance to infection with an otherwise relatively avirulent pathogen. Despite differences in experimental approach, the results reported in this paper tend to support such a concept.

If, as is reported by Berry and Smythe (1960), endotoxins contribute to the toxic manifestations of salmonellosis, then the close similarity of survivorship and survival time in animals infected with virulent SR-11, regardless of subsequent temperature exposure, might be the result of the formation of a smaller amount of endotoxin at 5° C than at 25° C. This argument would follow, since cold exposed mice are killed with less endotoxin than those maintained at normal temperatures. In line with this hypothesis, animals under optimal environmental conditions might support more rapid growth of certain virulent pathogens than animals in the cold. Perhaps infection with the avirulent SR-11-A results in more deaths in a shorter time at 5° C than at 25° C because the lethal level of endotoxin accumulates sooner at the former temperature. The defenses of the mouse might be sufficiently impaired by cold to permit a steady in vivo proliferation of the bacteria which ultimately leads to death, in contrast with the events in animals under more normal environmental temperatures.

The results with Staphylococcus aureus infections seem to fit into the interpretations suggested above. The relationship between virulent and avirulent S. aureus parallels closely the findings with salmonellosis. Additional studies will be required, however, before the role of cold in active infections can be clearly interpreted.

From our results it appears that the host is able to overcome certain effects of endotoxin within the first 6 to 12 hours of administration. Reports available in the literature suggest too that host

PREVITE AND BERRY

response to endotoxin is prompt and dramatic (Conti et al., 1961; Berry and Smythe, 1961). The period of sensitization to lipopolysaccharide by cold exposure (Tables Va and Vb) is paralleled by the time at which a drop in body temperature occurs following cold exposure and/or endotoxin poisoning (Table VI). Whether the reduction in rectal temperatures is the cause or result or merely reflects metabolic changes that account for sensitization remains unanswered. However, the latter view seems more likely in view of recently obtained but yet unpublished data from this laboratory.

Sensitization of mice to bacterial endotoxin by cold exposure may be related to adrenocortical function. If the stress of cold results in an initial hyperactivity of the gland, an alarm reaction would occur as part of a general adaptation syndrome (Selye, 1955). Adrenal activity is said to level off after an initial rise following exposure to cold (Heroux and Hart, 1954a; Schonbaum, 1960), while the low cholesterol content of the adrenals of room temperature exposed mice 17 hours after endotoxin administration has been interpreted as being the result of an earlier hyperactivity of these glands (Berry and Smythe, 1961). In view of the protective cortical hormones afford against endotoxin (Brooke et al., 1959), cold stress (Heroux and Hart, 1954b), or both of these factors combined (Table VII), the following postulate does not seem unreasonable. The greater resistance to endotoxin of cold-acclimatized mice compared to non-acclimatized animals may involve a greater capacity of the adrenal of the former to release protective corticoids at the proper time. In rats exposed to 5° C, the maximal activity of the adrenal cortex as shown by P³² uptake occurs at 2 hours after exposure (Rossiter and Nicholls, 1957). Thus it may be that an initial depletion of corticoid reserves occurs in the cold exposed mouse prior to the time at which these hormones are needed for protection against endotoxin. "Functional adrenalectomy" due to temperature stress, therefore, may be responsible for sensitization of cold exposed mice to endotoxin, just as surgical adrenalectomy has been reported to do so in rats (Brooke et al., 1959).

While the present report emphasizes adrenal cortical involvement in protection or sensitization to endotoxin following cold exposure, it is our opinion that more data must be gathered concerning other facets of endocrine involvement, metabolism, and host defense

VIRULENCE AND BACTERIAL INFECTION

mechanisms before definitive and final statements can be made concerning this problem.

SUMMARY

Mice maintained in individual compartments without bedding following infection with an avirulent strain of either Salmonella typhimurium or Staphylococcus aureus are more susceptible when exposed continuously to 5° C than they are when exposed to 15° C or to 25° C. These differences are not observed when virulent strains are used, while acclimatization to cold for two weeks fails to alter the response to the avirulent organisms. Mice kept at 5° C after injection are sensitized 250-fold to pasteurized S. typhimurium and about 10-fold to lipopolysaccharide derived from Serratia marcescens compared to control animals housed at 25° C. Mice given an LD₇₅ dose of lipopolysaccharide and placed at 5° C for 12 hours before transfer to 25° C are as susceptible to the endotoxin as mice kept continuously in the cold. Conversely, mice given the same dose and retained at 25° C for 6 or 12 hours before placing them at 5° C are almost as resistant as mice kept continuously at 25° C. The period of sensitization to lipopolysaccharide following cold exposure was paralleled by the time at which a drop in body temperature occurred following the low temperature stress and/or endotoxin poisoning.

Protection was afforded the cold exposed mice against endotoxin poisoning by exogenously administered cortisone acetate, while 8 units of ACTH enhanced the lethal effects of the toxin. The adrenal response of the host to temperature stress seems to be of paramount significance in determining the sensitization to lipopolysaccharide.

PREVITE AND BERRY

LITERATURE CITED

1. Barnett, S. A., E. M. Coleman, and B. M. Manly. 1959. Oxygen consumption and body fat of mice living at -3° C. *Quart. J. Exp. Physiol.* 44: 43-51.
2. Berry, L. J., and D. S. Smythe. 1959. Effects of bacterial endotoxin on metabolism. II. Protein-carbohydrate balance following cortisone inhibition of intestinal absorption and adrenal response to ACTH. *J. Exp. Med.* 110: 407-418.
3. Berry, L. J., and D. S. Smythe. 1961. Effects of Bacterial endotoxins on metabolism. IV. Renal function and adrenocortical activity as factors in the nitrogen excretion assay for endotoxin. *J. Exp. Med.* 114: 761-778.
4. Berry, L. J., and D. S. Smythe. 1960. Some metabolic aspects of host-parasite interactions in the mouse typhoid model. *Ann. N. Y. Acad. Sci.* 88: 1278-1286.
5. Berry, L. J., D. S. Smythe, and L. G. Young. 1959. Effects of bacterial endotoxin on metabolism. I. Carbohydrate depletion and the protective role of cortisone. *J. Exp. Med.* 110: 389-405.
6. Brooke, M., E. H. Kass, and O. Hechter. 1959. Protective effect of steroids against bacterial endotoxin. *Fed. Proc.* 18: 560.
7. Conti, C. R., L. E. Cluff, and E. P. Scheder. 1961. Studies on the pathogenesis of staphylococcal infection. IV. The effect of bacterial endotoxin. *J. Exp. Med.* 113: 845-860.
8. Croxton, F. E. 1959. Elementary statistics with applications in medicine and the biological sciences. New York, Dover Publications, Inc., pp. 267-283.
9. Duffy, B. J., and H. R. Morgan. 1951. ACTH and cortisone aggravation or suppression of the febrile response of rabbits to bacterial endotoxin. *Proc. Soc. Exp. Biol. Med.* 78: 687.

VIRULENCE AND BACTERIAL INFECTION

10. Girone, J. A. 1962. The effects of low temperature on bacterial infection. *Mendel Bulletin* 34: 5-10. Villanova University Press, Villanova, Pa.
11. Halberg, F., and W. W. Spink. 1956. The influence of brucella somatic antigen (Endotoxin) upon the temperature rhythm of intact mice. *Lab. Investigation* 5: 283-294.
12. Heroux, O., and J. S. Hart. 1954a. Adrenal cortical hormone requirement of warm and cold acclimated rats after adrenalectomy. *Am. J. Physiol.* 178: 449-452.
13. Heroux, O., and J. S. Hart. 1954b. Cold acclimation and adrenal cortical activity as measured by eosinophil levels. *Am. J. Physiol.* 178: 453-456.
14. Houser, E. D., and L. J. Berry. 1961. The pathogenesis of staphylococcus infections. I. The use of diffusion chambers in establishing the role of staphylococcal toxins. *J. Infect. Dis.* 109: 24-30.
15. Kulka, J. P. 1961. Vasomotor or microcirculatory insufficiency: Observations on nonfreezing cold injury of the mouse ear. *Angiology* 12: 491-506.
16. Muschenheim, D., D. R. Duerschner, and J. D. Hardy. 1943. Hypothermia in experimental infections. III. The effect of hypothermia on resistance to experimental pneumococcus infection. *J. Infect. Dis.* 72: 187-196.
17. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 27: 493-499.
18. Rossiter, R. J., and D. Nicholls. 1957. Phosphorous metabolism of the adrenal gland of rats exposed to a cold environment. *Revue Canadienne de Biologie* 16: 249-268.

PREVITE AND BERRY

19. Schneider, H. A. 1949. Nutrition of the host and natural resistance to infection. IV. The capability of the double strain inoculation test to reveal genetically determined differences in natural resistance to infection. *J. Exp. Med.* 89: 529-539.
20. Schneider, H. A., and N. Zinder. 1956. V. An improved assay employing genetic markers in the double strain inoculation test. *J. Exp. Med.* 103: 207-224.
21. Schönbaum, E. 1960. Adrenocortical function in rats exposed to low environmental temperatures. *Federation Proc.* 19: 85-88.
22. Selye, H. 1955. Stress and disease. *Geriatrics* 10: 253-261.
23. White, C. 1952. The use of ranks in a test for significance for comparing two treatments. *Biometrics* 8: 33-41.

DISCUSSION

BLAIR: I was particularly interested in the development of the hypothermia of the *Serratia* endotoxin. I wonder if here again we aren't faced with the problem of the species differences in experimental work of this nature. Maxwell, several years ago, using dogs -- I am sorry, you used *Serratia* endotoxin also -- uniformly obtained hyperpyrexia, and his animals died in the classic picture of septic shock with a high fever.

PREVITE: In response to that, I would say that mice seem to be peculiar animals in that respect. Although I don't profess to have tremendous knowledge concerning the effects of endotoxin, from what I have read, it seems as if the mouse is one of the few animals that responds to this poison with a lowered body temperature. Most animals, including man and rabbits, respond with hyperthermia. These divergent responses again point out significant species differences.

VIRULENCE AND BACTERIAL INFECTION

BLAIR: I ask what the condition of the mice was at the 5° C exposure. That is, after development of hypothermia, whether they were lethargic or comatose or not just before they died?

PREVITE: You are referring to the control mice placed at 5° C?

BLAIR: No, the ones that were infected.

PREVITE: Measurements weren't made following infection; they were made following endotoxin injection. But these animals, after 6 to 12 hours with and LD₅₀ dose, were pretty sick. They did not move in the cage. Within eight hours, one could practically predict by its rectal temperature that endotoxin-poisoned, cold stressed mouse which was going to die or survive. Animals whose rectal temperatures dropped to as low as 29.5° C always died. And those animals whose temperatures were 33° C to 34° C or higher usually survived what was potentially a lethal dose of endotoxin.

SCHMIDT: But in your third to the last slide, I think you were showing us the effects of endotoxins on the temperature of the animal. I may be mistaken, but as I recall, you were indicating for your control animals at 25° C that the rectal temperature was 37.4° C, and then, as the hours progressed, the animals at 5° C showed somewhat lowered rectal temperatures in the range of 36° C, and then you said that at 24 hours, this difference had been overcome.

PREVITE: The difference between the 25° C group and the 5° C group?

SCHMIDT: Yes, but what I think is significant is that the ones at 25° C were now approximately 1° C lower in rectal temperature than they were when they started. In other words, you got a fluctuation of 1° C normally.

PREVITE: If you take the average without considering the deviation, yes, but if you compare these statistically, because of the overlap from one mouse to another, there is no significant

PREVITE AND BERRY

difference after 24 hours. Therefore, my findings would essentially agree with those of Dr. Miya. That is, within 24 hours, the body temperature of control animals exposed to 5° C returns to the normal range.

SCHMIDT: You have essentially shown us that you can take a group of ten animals and leave them at 25° C, and their average rectal temperature is going to drop 1° C.

PREVITE: It is not one complete degree. It's a fraction of a degree. Again, if one compares the figures 37.8° C versus 36.6° C alone, they do seem to differ significantly. However, upon statistical analysis they are not significantly different because of the wide mean deviation and overlap between them.

BERRY: I would like to comment first on Mr. Schmidt's question. It is very relevant. It seems a little peculiar, when you look at the cold figures, to find temperature differences, but it may be just a statistical fluke. Mice show variations in temperature during the day, and particularly, with time of day. This is a well known fact. Around 4:00 a.m., or at least early morning, the temperature is typically a full degree lower than it is in the afternoon. This has been studied extensively by people interested in biological clocks. Now, with any group of mice, in order to establish a change in body temperature with time of day, it is necessary to work with large numbers of animals and to maintain, if possible, a constant environment. With a small sample of mice, it may be possible to get nearly a degree's temperature average difference just because of a sampling anomaly.

WALKER: I think you can get as much of a temperature difference as that and more in addition to that with just a little bit of activity.

BERRY: Yes, and you probably change their temperature with rectal probes.

WALKER: Wisconsin mice don't like a rectal probe at first, and they usually struggle, which will run the body temperature up. If you let them struggle and keep the probe there, you can follow his temperature climb. If you take ten mice in a group

VIRULENCE AND BACTERIAL INFECTION

and put them into the cage, the tenth mouse will have a temperature one degree higher than the first one just because of your reaching in and stirring these animals up.

PREVITE: Regarding that, I noticed that when a timer with a bell was used in order to leave the rectal thermistor probe in the cold exposed mouse for exactly 15 seconds, the stress of the ringing bell at the end of this period seemed to affect the rectal temperature measurement. Rectal temperatures were lower in mice for which a wristwatch was used to time the 15 second period of thermistor probe insertion. Apparently the sound of the bell served as a stress which could affect body temperature measurements.

BLAIR: Adolph at Rochester and Crisman at Stanford, in order to produce hypothermia in mice and rats, used no anesthesia. It was necessary to restrain the animals. If they were not restrained, no matter how long they were exposed in an environment of 0° C to 5° C, they do not achieve a hypothermia.

WALKER: What is meant by hypothermia? How low is it?

BLAIR: He has taken it down as low as 18° C or 15° C, but the strain on the mice and the initial activity causes elevation in the body temperature, but within several hours they are cooled down quite extensively, as opposed to the group which is allowed to run around in the cages. So the strain is important. This is related to the muscle activity you are talking about.

REINHARD: What was your statement relative to the genetics of the organism and the host-parasite relationship?

PREVITE: We noted that there is a difference between virulent and avirulent strains. The genetic constitution of the parasite can be very important in determining the outcome of the infection as modified by cold exposure.

REINHARD: In a homozygous physiologically standardized host?

PREVITE: I made no reference to the host at all, but I would

PREVITE AND BERRY

agree with you, certainly, the genetic constitution of the host can be just an important.

WALKER: I think we should not expect to see an effect with a very virulent strain or with a virulent virus in a very susceptible host. These are not the circumstances in which you can see small differences. It is very important to have the strain and host under the right circumstances; it seems to me we can then see an effect of temperature. It's ridiculous to expect a measurable effect under all circumstances. Much of the variation in the results reported from experimental work concerned with the effect of cold on infection can be explained, I think, by the variation in host and strain of infectious agent used in the experimental work.

TRAPANI: I am a little curious about something on which you might like to comment. I'm thinking about the effect of ACTH and corticoids, especially. We always think about their possible effect on the host. However, Dr. Miya's results showed that bacteria can change, too, with the animal. Is it possible that there is also an effect on the invading organism? Is there any other work which points to this?

MIRAGLIA: I will comment on this later on this afternoon.

PREVITE: The data I presented would give us little direct information regarding the role of adrenal corticoids and the in vivo infectious process. My work thus far with adrenal corticoids has been centered around the effect of these hormones on the response of mice to heat killed cells or commercially prepared endotoxin.

ENDOGENEOUS AND EXPERIMENTAL PERITONITIS AND BACTEREMIA IN HYPOTHERMIC MICE

G. Tunevall and T. Lindner

Central Bacteriology Laboratory
Box 177
Stockholm 1, Sweden

ABSTRACT

In experiments with mice, hypothermia at 22° C to 23° C, when maintained for more than 40 hours, almost invariably resulted in the emergence of bacteria in peritoneal fluid and blood. *Klebsiellae*, rapidly eliminated in normal mice, diminished in number only during a period of 2-4 hours, but thereafter increased again in hypothermic mice and caused a profuse bacteraemia. As antibacterial treatment reduced the incidence of peritonitis and bacteraemia without increasing the average survival time in hypothermia, the bacterial invasion is not likely to be of major importance for debilitation and death, but rather a concomitant phenomenon. In subsequent series of experiments, groups of mice were inoculated intraperitoneally with pneumococcal suspensions. The following observations were made: the increase in numbers of pneumococci in the blood was slower in hypothermic mice, the establishment of bacteraemia occurred later in such mice, and pneumonic alterations in the lungs were less common in hypothermic mice. In warming up of hypothermic mice, the events in all these respects were accelerated to equalize the eventual results in these animals with those of the normothermic controls.

I. Endogeneous Peritonitis and Bacteremia

The concept of "endogeneous bacteremia" has been created in connection with studies on the effect of irradiation and cortisone treatment of animals (Bennet et al., 1951; Berlin et al., 1952; Fallowfield, 1962; Gledhill and Rees, 1952; Hammond et al., 1954; Miller et al., 1950; Miller et al., 1952; Philipson and Laurell, 1958; Sanders et al., 1957). It is conceivable that this condition, generally appearing as an invasion of the blood stream by bacteria normally present in the intestine, may also take place in hypothermia, as this state is known to interfere with several mechanisms taking part in the anti-

TUNEVALL AND LINDNER

bacterial defense of the body, as antibody formation (Lindner and Tunevall, 1958) leucocytotic reaction (Fedor et al., 1958; Helmsworth et al., 1955; Villalobos et al., 1955), phagocytosis (Fedor et al., 1958) or local tissue reactions (Beyer, 1956; Sanders et al., 1957; Szilagyi et al., 1956). As early as 1897, Fischl observed fatal septicemia in rabbits subjected to chilling.

More recently, the possibility of bacteremia has been studied by Fedor et al. (1956) in dogs maintained at 28° C to 29° C for 6 to 12 hours. In such animals no endogenous bacteremia occurred, and the ability to clear the blood stream from injected bacteria was very slightly impaired. On the other hand, Billingham (1957) observed in extreme hypothermia an increased passage of bacteria through the endothelial lining of the gut. Having observed at autopsies of mice subjected to prolonged hypothermia the frequent occurrence of peritonitis and bacteremia, we have undertaken some experiments in order to study more closely the development of these conditions and to establish their importance for the mortality of mice kept in the hypothermic state. Further, as a complement to this study, an investigation of the ability of hypothermic mice to eliminate injected bacteria from the blood stream was undertaken.

METHODS

Inbred albino mice weighing 15 to 20 gm were used for the experiments. The procedure for bringing mice into a controlled hypothermic state and maintaining them there has been described in a previous paper (Lindner and Tunevall, 1958) and will not be repeated here in detail. Briefly, the animals were pre-treated with chlorpromazine-HCl (Hibernal) and ethyl-(1-methyl-butyl)-malonyl-carbamide-Na (Nembutal) before being immersed into a water bath where they were, under administration of oxygen, kept at a body temperature of 22° C (registered rectally).

Intravenous injections were made into the dorsal vein of the tail with a No. 20 "Record" cannula on a syringe of the tuberculin type.

PERITONITIS AND BACTEREMIA IN MICE

For bacterial counts, blood samples of 0.02 ml were drawn with the same type of syringe prefilled with 0.18 ml of 0.85 per cent NaCl solution after cutting the dorsal vein of the tail thoroughly cleaned with 70 per cent ethyl alcohol. The resulting 1:10 dilution was spread over a 3 per cent horse blood agar plate. After incubation overnight at 37° C, the bacterial growth was roughly estimated as "(+)" (1-5) colonies "+ " (6-50), "++" (51-500), or "+++" (more than 500). In the elimination studies, appropriate dilutions of the samples were made to permit a precise count.

Fecal specimens were taken from the rectum with a 2 mm platinum loop and spread on plates as above.

At autopsy, fecal specimens were taken as described above, blood samples from heart puncture were cultured as was described for the tail blood, and peritoneal fluid was collected with a cotton swab from the abdominal cavity and directly spread on a blood agar plate.

RESULTS

Influence of Hypothermia on the Intestinal Flora

As the main source of endogenous bacteremia is likely to be the intestine, the composition and density of the aerobic fecal flora were studied after various periods of hypothermia. The results of fecal cultures are presented in Figure 1a; point of time is represented by numbers of cultures ranging from 109 to 29. Within the coliform group, the proportions of E. coli, A. aerogenes, and Paracolo-bacteria were not significantly altered during the experiments. Small numbers of bacteria of other species not represented in the figure were observed but are disregarded here for simplicity. It is evident that the intestinal flora as represented by fecal cultures was not altered significantly in prolonged hypothermia.

TUNEVALL AND LINDNER

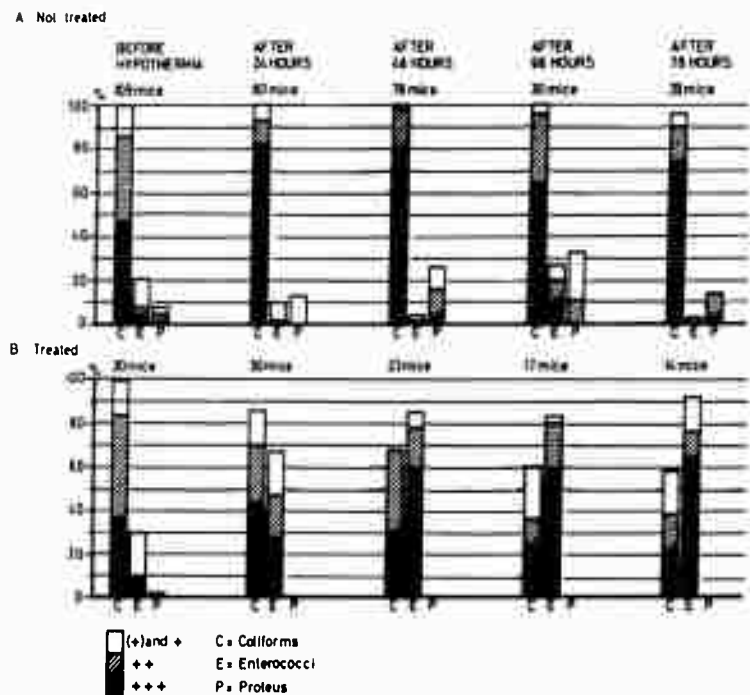


Figure 1. Results of fecal cultures after different periods of hypothermia.

The Development of Bacteremia in Hypothermic Mice

In four consecutive experiments, in which 34 mice not anti-bacterially treated were used, repeated blood cultures were made after various periods of hypothermia. The results are reported separately for the four experiments in Figure 2. In 8 mice bacteremia occurred after only 24 to 29 hours of hypothermia. After 48 to 54 hours, the number of bacteremic animals had increased to 14. After 78 hours, only three mice remained alive and free from bacteria in their blood, and of these, two mice soon succumbed in a bacteremic state.

The same events are described for all four experiments together in Figure 3, this time with different bacterial species re-

PERITONITIS AND BACTEREMIA IN MICE

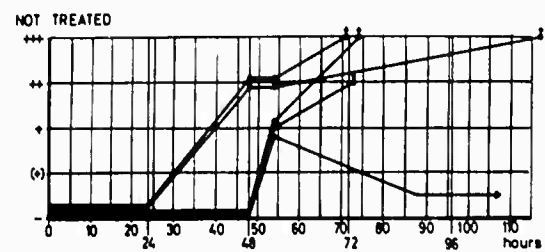
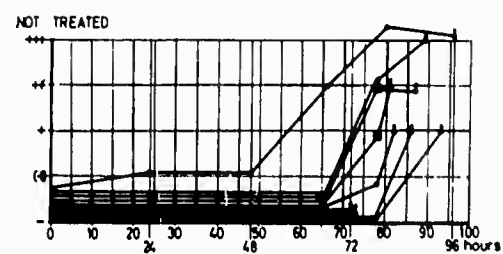
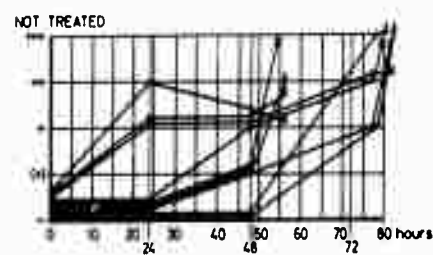
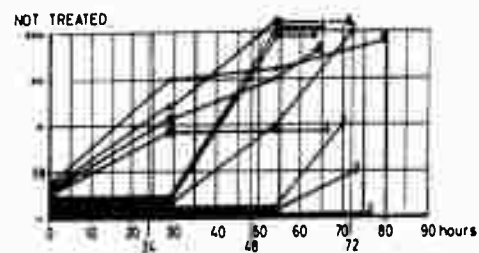


Figure 2. Results of blood cultures made after various periods of hypothermia on 39 mice not antibacterially treated. (+) = 1-5 colonies, + = 6-50, ++ = 51-500, +++ = more than 500.

TUNEVALL AND LINDNER

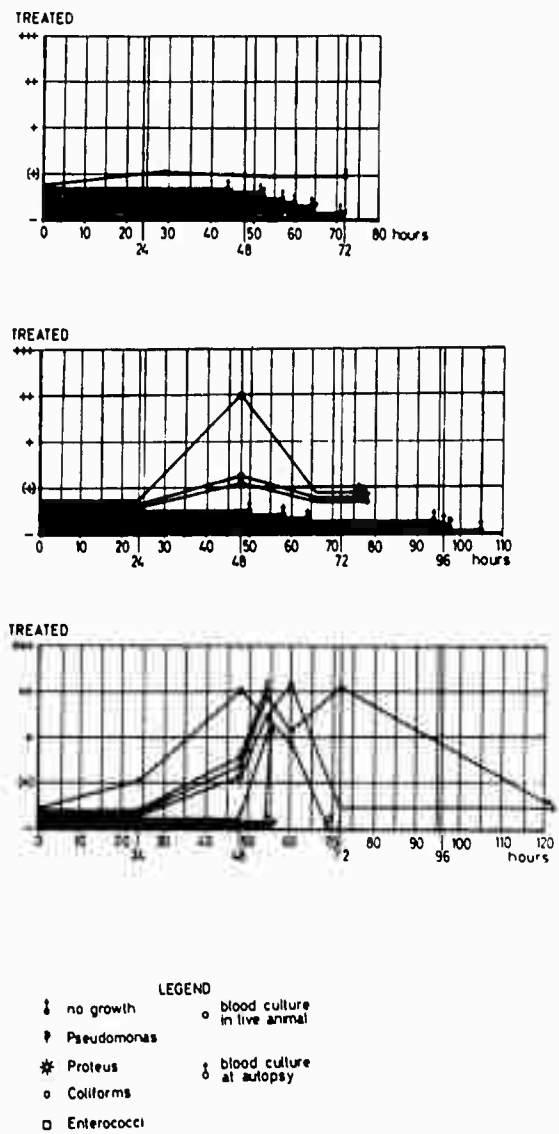


Figure 2 (cont.). Results of blood cultures made after various periods of hypothermia on 28 antibacterially treated mice.

PERITONITIS AND BACTEREMIA IN MICE

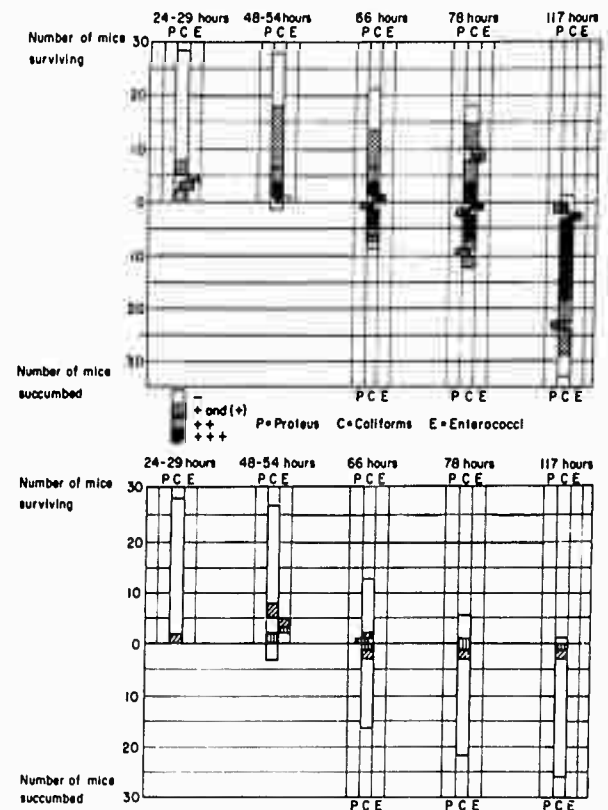


Figure 3. Results of blood cultures in 62 mice, 28 of which were treated antibacterially. The height of the columns above zero line indicates the number of living mice; under this line that of dead mice. Top = 34 mice not treated. Bottom = 28 mice treated with sulpha and streptomycin.

ported separately. In no case was an organism found in the blood which had not been present in feces at the onset of bacteremia.

Peritonitis and Bacteremia in Sacrificed and Succumbed Mice

Cultures from peritoneal fluid and from blood were made in 30 mice sacrificed in good condition and in 62 mice which had died

TUNEVALL AND LINDNER

A. Not treated

Mice sacrificed after 41 - 48 hours 49 - 60 hours

Number of mice	17			3		
Mice with pos. cultures	4			1		
Thereof in perit./blood	2/3			0/1		
Organism	Perit.	Blood		Perit.	Blood	
		+++	+		+++	+
E. coli	2	2			1	
Paracoli	2	1	1			

Mice succumbed after 41 - 48 hours 49 - 96 hours

Number of mice	22			40		
Mice with pos. cultures	18			38		
Thereof in perit./blood	17/13			36/35		
Organism	Perit.	Blood		Perit.	Blood	
		+++	+		+++	+
E. coli	12	8	4	31	20	8
Paracoli	1	1		3	1	1
A. aerogenes	2			2	2	1
Alcalig. faecalis	3	1		2	1	
Proteus	3	1	1	4	3	3
Enterococci		1		3		
B. mesentericus				1	1	

B. Treated with sulpha + streptomycin

Mice succumbed after 41 - 48 hours 49 - 105 hours

Number of mice	1			25		
Mice with pos. cultures	0			6		
Thereof in perit./blood	0/0			6/3		
Organism	Perit.	Blood		Perit.	Blood	
		+++	+		+++	+
E. coli				5		3
Enterococci				1		

Figure 4. Bacterial findings in peritoneal fluid and in blood after various periods of hypothermia. Less than 10,000 bacteria per ml blood is denoted as +, more than 10,000 as +++.

PERITONITIS AND BACTEREMIA IN MICE

A. Identic findings from blood and peritoneal fluid: B + P
Findings from peritoneal fluid when sterile blood: P
Findings from blood when sterile peritoneal fluid: B

	B + P	P	B
E. coli	27	2	1
Alcaligenes faecalis	1	1	
Proteus		1	4
Enterococci	2		
B. mesentericus	1		
E. coli + paracoli		1	
E. coli + A. aerogenes	3		
E. coli + Alcalig.faecalis	1	2	
E. coli + Proteus	4	2	
Paracoli + Alcalig.faecalis	—	—	1
Total	39	9	6

B. Partial identity between peritoneal and blood findings.

		Recovered from blood	
		E. coli	E. coli + paracoli
From	E. coli + paracoli	1	
peri-	E. coli + A. aerogenes	1	
toneal	E. coli + enterococci	1	
fluid	Paracoli + Proteus		4

Figure 5. Bacterial species recovered from blood and peritoneal fluid in mice sacrificed or succumbed after prolonged hypothermia.

after various periods of hypothermia. Inten animals sacrificed after 24 or 40 hours, the peritoneal fluid as well as the blood was sterile. The findings from the remaining 82 mice are presented in Figure 4, where colony counts below 500 are marked "+" and those above 500 "+++".

From the table it is evident that in mice sacrificed in good condition, peritonitis and bacteremia are fairly infrequent when compared to their occurrence in spontaneously dead animals after long or short periods of hypothermia. In the group which succumbed early bacteria were present in peritoneal fluid in 5 instances without oc-

TUNEVALL AND LINDNER

curing in blood, but in blood only once without being present in the peritoneum.

The degree of correspondence between the bacterial findings from peritoneum and those from blood is visible from Figure 5a. Full conformity existed in 39 cases, whereas in 9 mice, bacteria were present in peritoneal fluid but not in blood, and in 6 instances in blood without being present in peritoneum. Partial conformity existed in the remaining 7 cases.

Influence of Hypothermia on the Elimination of Bacteria From the Blood

In a number of preparatory experiments various numbers of E. coli of strains emanating from mouse feces were injected intravenously into normal mice. After different periods, the blood count of live bacteria was measured. The results are reported in Figure 6. Regardless of the number of bacteria injected, the elimination was rapid during the first 15 minutes, bringing down the bacterial counts to about one tenth of the numbers found 4 minutes after the injection. In one experiment, where the first count was made only one minute after the injection, the reduction was equally rapid from this time. After the fifteenth minute, the elimination rate was reduced. This varied somewhat between different experiments, but always resulted in a complete or almost complete elimination. All animals survived.

Considering the frequent occurrence in the blood of E. coli after prolonged hypothermia, some other organism not usually found in endogenous bacteremia had to be chosen for the elimination studies on hypothermic mice. Hence, the following experiments were performed with Klebsiella pneumoniae in a constant dose of 4×10^6 bacteria. The following groups, each containing 5 mice, were studied: normal mice; mice having received premedication but not chilled; mice made hypothermic 5 minutes after the injection of bacteria, 2 hours before the injection, 24 hours (2 groups), and 48 hours (2 groups) before same injection. The results are presented graphically in Figure 7.

PERITONITIS AND BACTEREMIA IN MICE

log number of bacteria
per ml blood

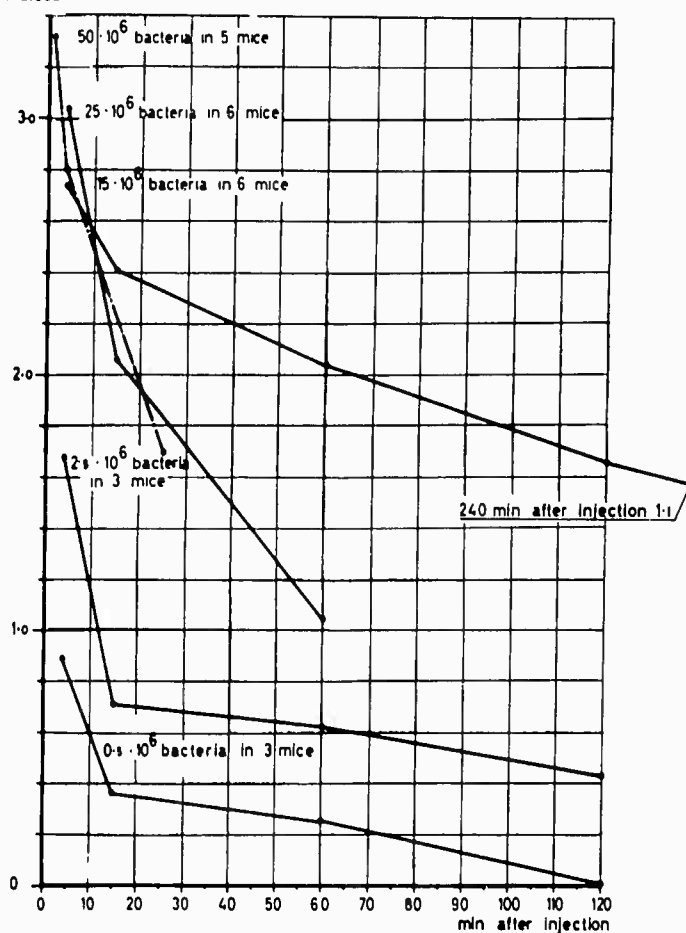


Figure 6. Results of blood cultures in mice at different times after the intravenous injection of *E. coli* suspensions. The points denote logarithmic means. Numbers of mice in each group and number of bacteria injected are given in the figure.

In normal animals the initial elimination was rapid in this series also, leading to complete disappearance of bacteria from the blood. In hypothermic animals the 4 minute counts gave higher numbers which increased with the length of the previous period of hypothermia. Further, the elimination process was interrupted after 2 or

TUNEVALL AND LINDNER

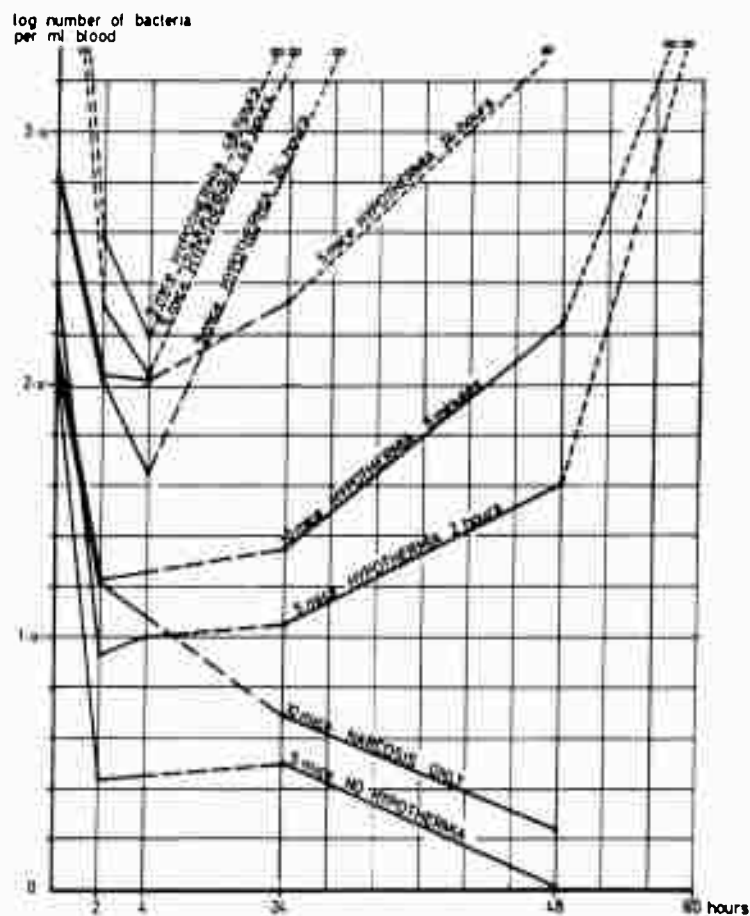


Figure 7. Results of blood cultures in mice at different times after the intravenous injection of $4 \cdot 10^6$ Klebsiellae. The points represent logarithmic means. The numbers of mice in the groups and the length of hypothermia preceding the injection are given in the figure.

4 hours. The level of the minimum colony count also varied with the length of the preceding period of hypothermia. Thereafter an increase of the bacterial counts occurred, ending in a state with innumerable colonies also from the highest blood dilutions. This took place after 72 hours in mice hypothermic 2 hours prior to

PERITONITIS AND BACTEREMIA IN MICE

injection; after 48 hours in those cooled down 24 hours before; and after 24 hours in mice hypothermic for 48 hours before injection of bacteria.

The Influence on Endogeneous Bacteremia of Antibacterial Treatment

The experiments now described indicate that bacteremia or peritonitis, or both, occurred almost regularly in mice subjected to prolonged hypothermia, though they were more frequent in mice which succumbed in a hypothermic state than in animals sacrificed in good condition after the same time for hypothermia. The significance of these observations is not clear. Bacteremia and peritonitis may be the dominating cause of debilitation and death, or it may be a concomitant of other changes due to hypothermia. In order to shed some light on this problem, experiments were arranged in which hypothermic mice were treated with a combination of sulpha drugs and streptomycin during hypothermia. This combination was found in preliminary in vitro tests to be most effective against the bacterial strains usually found in bacteremia.¹

The influence of such treatment on the intestinal flora is presented in Figure 1b, from which it can be seen that a shift from a dominance of coliform bacilli to a prevalence of enterococci was effected.

In several of the previously described experiments on the development of bacteremia in prolonged hypothermia, groups of antibacterially treated mice were run in parallel. The results are seen in Figure 4b and Figures 2b and 3b. It is evident that bacteremia was largely prevented by the treatment. The survival time, however, was not prolonged; not even in the experiment where the untreated animals were given blind injections to compensate for the stress which repeated injections per se might mean for the treated mice.

¹ The preparations and doses used were: "Sulfodital" (Sulfonazole 37 p. c., Sulfadiazine 37 p. c., Sulfamerazine 26 p. c.) 100 mg/kg/day; subcutaneously divided into two doses; and streptomycin 40 mg/kg/day, in one dose.

TUNEVALL AND LINDNER

DISCUSSION

Observations of other investigators, corroborated in the present study by findings to be discussed below, make us accept the view of the intestinal flora as the source of endogeneous bacteremia in prolonged hypothermia. The ability of intestinal bacteria to invade the rest of the body during the hypothermic state might be due to a change of this flora itself. The absence of food intake, the slowing down of intestinal motility, and the low temperature seem likely to effect such a change. However, as shown in Figure 4, no major alteration of the composition occurred. It must be stated in this connection that our studies were restricted to aerobic organisms.

By repeated blood cultures in hypothermic mice, bacteremia was found to be an almost constant result of deep and prolonged hypothermia, as shown in the Figures 2 and 3. It should be noted that no bacterial type was ever demonstrated in the blood without having been found previously in the intestine. Intestinal and blood findings were not compared by serological typing but in biochemical reaction of *E. coli* strains and in Dienes' identity test for *Proteus bacilli* (Dienes, 1946) full conformity was found between every pair of strains tested.

As to the route of the bacterial invasion from the intestine, Figures 4 and 5 indicate that in sacrificed or succumbed mice, bacteria were more often found in peritoneal fluid without being present in the blood than in blood without being found in the peritoneal cavity. Thus, the invasion often started with a peritonitis but seemed also to take place directly into the lymph or blood stream. Hammond et al., (1954) refer to experiments where bacteria were found to penetrate the intestinal wall and to reach the mesenteric lymph nodes in X-irradiated mice.

A demonstrable occurrence of bacteria in the blood can, apart from an abnormal import as suggested by Billingham (1957), be due to a diminished ability of the organism to eliminate bacteria from the blood stream. Our experiments reported in Figure 7 demonstrate such an impaired elimination; the more it was decreased, the longer

PERITONITIS AND BACTEREMIA IN MICE

the hypothermic state existed. They also show that the intravenous injection of bacteria which are normally eliminated from the blood without any consequences for the animal results in a profuse bacteremia in mice in deep hypothermia. We have not yet had the opportunity to investigate whether this disagreement with Fedor's (1958) results are due to differences as to the kind of animal, the degree of hypothermia, or its duration, but it may be mentioned that the observations of Gowen and Friou (1961) in dogs tally with our results in mice.

The observation that peritonitis and bacteremia are more frequent in spontaneously dead mice than in animals sacrificed in good condition after the same time of hypothermia may be interpreted in two ways. The generalized infection may be a dominating cause of death, or it may be a concomitant phenomenon to other injuries caused by hypothermia. In the former case it should be possible to diminish the mortality of hypothermic mice with antibacterial treatment. Such treatment was given to groups of hypothermic mice.

The treatment (streptomycin + sulpha) effected a pronounced change in the aerobic intestinal flora from a preponderance of the *Escherichia-Aerobacter* group to a dominance of enterococci. The latter species, however, did not replace the Gram-negative rods as blood invaders. Generally, no bacteremia was observed in treated animals. However, the average survival time in prolonged hypothermia was not increased. Thus, bacteremia seems not to be a determining factor in the debilitation and death of hypothermic mice. These observations coincide, for example, with those recorded in irradiated mice by Laurell et al. (1958) and Fallowfield et al. (1962), but differ from the results of Miller et al. (1952).

II. Experimental Peritonitis and Bacteremia

Many studies have dealt with the effect of low temperature on induced infection. The results have probably been contradictory be-

TUNEVALL AND LINDNER

cause of differences in the choice of experimental animals and infecting micro-organisms, and also of the mode of exposure to cold. Thus, in one type of study animals have been exposed to cold under conditions that provoke stress reactions, and chilling has generally been found to increase the susceptibility. In 1878, Pasteur found that chilled fowl became susceptible to anthrax. Increased susceptibility to trypanosomes at low environmental temperature has been seen in guinea pigs (Kligler and Weitzmann, 1926); in rats (Kligler, 1927) and mice (Kligler and Olitzki, 1931) to *Bac. enteritidis*; and in rabbits to streptococci (Carpano, 1926) and to syphilis (Longhin et al., 1957). Other such experiments have shown lowered resistance of chilled guinea pigs to pseudotuberculosis but not to pneumococci (Burgers, 1929), indicating the importance of the choice of infecting organism is important. This was also borne out by studies of Previte and Berry (1962) in which chilled mice were abnormally sensitive to relatively avirulent *Salmonellae* and staphylococci but normally susceptible to virulent strains. It is noteworthy that Bruneau and Heinbecker (1944) found that in dogs inoculated subcutaneously with β -streptococci, cooling reduced the local inflammatory reaction and arrested bacterial growth. On removal from the cold, however, both these processes were abnormally accelerated, indicating that prolonged chilling had in the long run lowered the resistance.

In another type of experiment, body temperature has been lowered in a way which avoids stress reactions. The results have been different in that infection with virulent Type I pneumococci in rabbits was not changed by hypothermia, whereas infection with normally avirulent Type III pneumococci became lethal in hypothermic animals (Muchenheim et al., 1943). Similar observations as to the result of infection with pneumococci of low virulence in rabbits were made by Sanders et al. (1957), but they also found a decrease of the survival time in virulent infection. On the contrary, Wotkyns et al., (1958) working with mice found a longer survival time in hypothermic animals with Type III pneumococcal peritonitis suggesting defense mechanisms. Similar observations on rats with peritonitis were made by Balch et al. (1955), and on rabbits with staphylococcal sepsis by Grechishkin (1956).

PERITONITIS AND BACTEREMIA IN MICE

EXPERIMENTAL

Method

Pneumococcal infection was induced by intraperitoneal (i.p.) injection of broth cultures in doses which were previously found to cause infection of a desired course and massivity in normal mice.

Results

In preliminary experiments for stating optimal conditions for the following study, hypothermia was found generally to prolong the average survival time of mice after their inoculation (i.p.) with pneumococci. The results of such an experiment are reported in Figure 8.

In all, 32 mice were inoculated (i.p.) with 20,000 pneumococci Type III, while six mice were kept as controls. About three hours later, 16 inoculated mice and the controls were made hypothermic. The remaining 16 inoculated mice were kept normothermic. After about 49 hours, surviving hypothermic mice were warmed up. Cultures from heart blood were made from succumbed mice as soon as we were sure they were dead.

The average survival time in the hypothermic group was 36 hours as opposed to 16 hours in the normothermic group and 59 hours for five out of six hypothermic controls not inoculated; the sixth animal in this group survived. In the inoculated hypothermic group, three mice lived to be warmed up, but died during or soon after this procedure. Pneumococci were found in peritoneal fluid of all inoculated mice. In heart blood they were abundant in all normothermic mice, but only in six hypothermic ones, whereas three had pneumococci in moderate numbers and seven mice had even less.

It should be added that among five inoculated hypothermic mice and five not inoculated ones which sustained hypothermia

TUNEVALL AND LINDNER

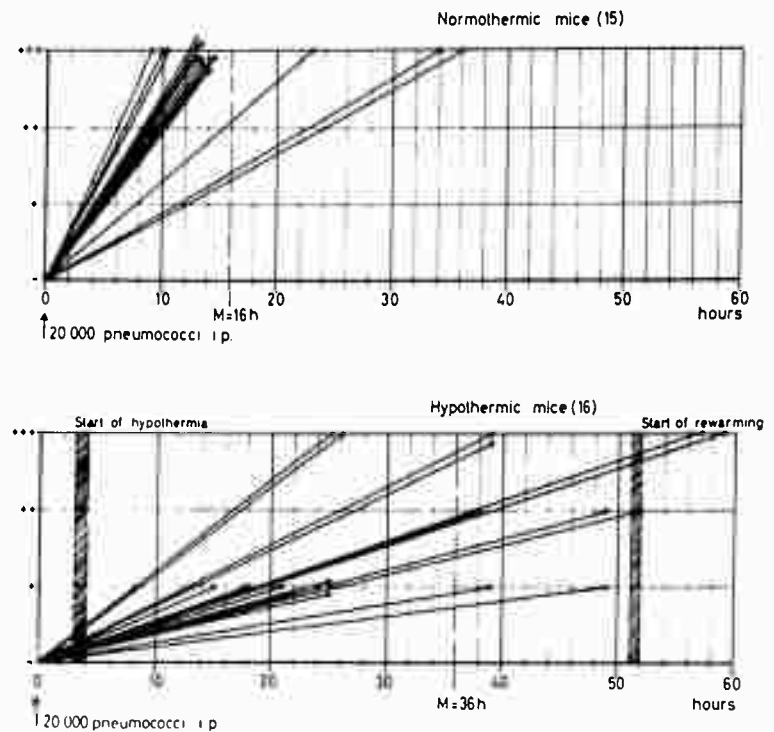


Figure 8. Results of post mortem blood cultures in normothermic mice and hypothermic ones given 20,000 pneumococci Type III intraperitoneally. + = 1-50 colonies, ++ = 51-500, +++ = more than 500.

for 46 hours or more, seven animals had coliform bacteria in blood and/or peritoneum.

The first experiment was only semi-quantitative and indicated just the final result of the infection, because post mortem cultures only were made. In the following study the events were followed more closely.

In the experiment reported in Figure 9, 15 mice were made hypothermic while another 15 received the pretreatment only. All mice were inoculated (i.p.) with 200,000 Type II pneumococci eight

PERITONITIS AND BACTEREMIA IN MICE

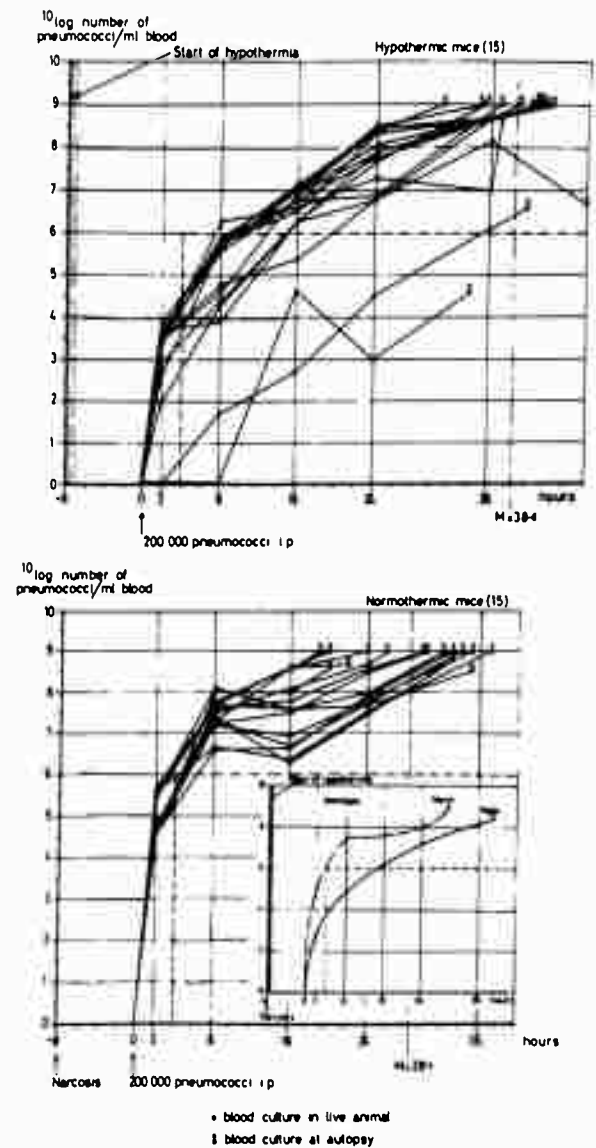


Figure 9. Results of intra vitam and post mortem blood cultures in hypothermic mice and normothermic ones given 200,000 pneumococci Type II intraperitoneally. Average curves inserted below.

TUNEVALL AND LINDNER

Hours after inoculation	M ¹⁰ log nr of pneumococci ± mean error of means		Diff.	t	P
	Hypothermic	Normothermic			
2	2.91 ± 0.33	5.00 ± 0.12	2.09	6.15	<0.001
8	4.71 ± 0.45	7.50 ± 0.11	2.79	6.18	<0.001
16	6.25 ± 0.31	7.63 ± 0.22	1.38	3.73	<0.001

Figure 10. Number of pneumococci in blood at different times after i. p. inoculation in hypothermic and normothermic mice. The figures are given as ¹⁰logs, with mean errors of the means. Differences, t-values in Student's test, and P-values are also given.

hours afterwards. The hypothermic mice were kept in this state during the whole experiment. In both groups blood counts were made 2, 8, 16, 24, and 36 hours after inoculation. Succumbed mice were autopsied immediately and bacterial counts were then made from heart blood.

As seen from the figure, bacteremia increased more slowly in the hypothermic mice. This also presented a longer survival time; 38.8 ± 1.0 hours as against 28.7 ± 1.6 hours for the normothermic ones (t-value 5.5, $P < 0.001$). The average numbers of pneumococci per ml blood in the two groups after 2, 8, and 16 hours are given in Figure 10 together with results of probability calculations. The formula used for testing the significance of differences is:

$$t = \frac{M_x - M_y}{\sqrt{\frac{(\bar{x} - x)^2}{n_x} + \frac{(\bar{y} - y)^2}{n_y}}}$$

From the figures in the table, the slower increase on the bacterial numbers among hypothermic mice is verified. After the 16th hour, the differences are no more significant.

PERITONITIS AND BACTEREMIA IN MICE

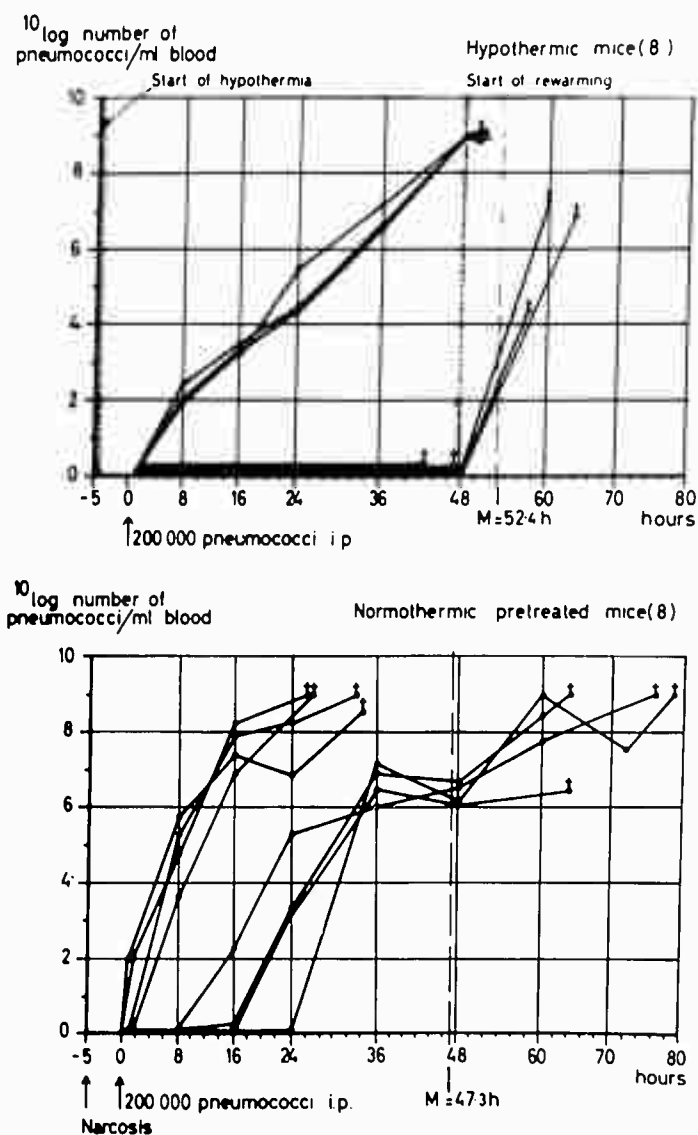


Figure 11. Results of inter vitam and post mortem blood cultures in hypothermic mice and normothermic ones given 200,000 pneumococci Type II intraperitoneally. Markings as in Figure 2.

TUNEVALL AND LINDNER

It was interesting to note that hardly any pneumonic lesions were found in the hypothermic group, but in five normothermic mice all with a survival time of 30 hours or more.

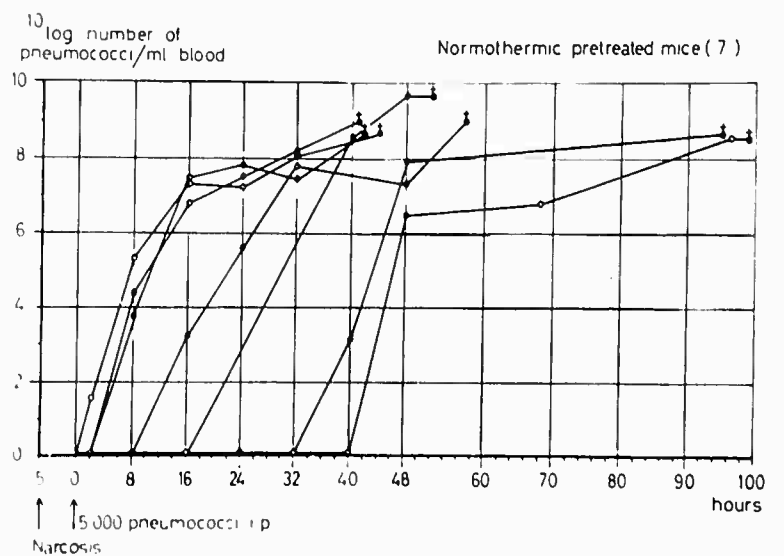
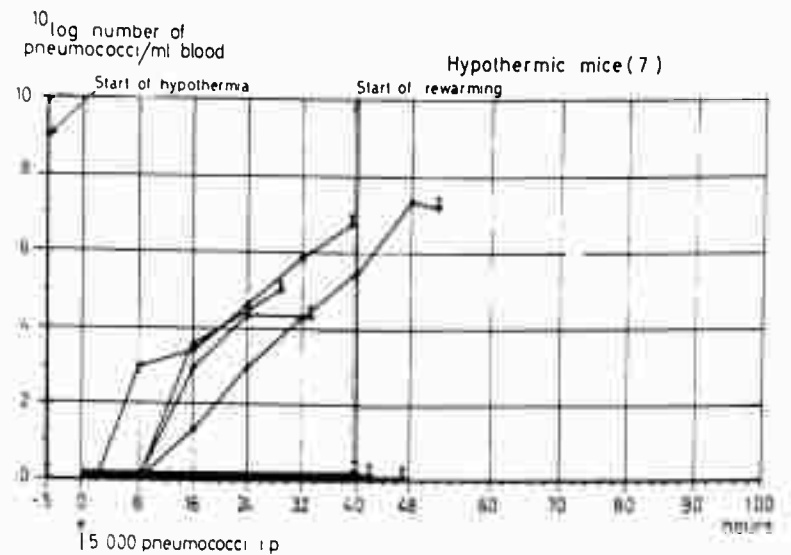
In the previous experiment the inoculation resulted in bacteremia which was always established after two hours in the normothermic group. In the hypothermic group, however, two animals had sterile blood after two hours and one of them after eight hours. In order to investigate if bacteremia could be regularly postponed by hypothermia, a less massive infection was induced in the following experiments, as the propagation of such an infection into the blood stream seemed more likely to be influenced by the hypothermic state.

In one of these experiments, presented in Figure 11, the hypothermic as well as the normothermic group contained eight mice. Two hundred thousand pneumococci Type II were inoculated (i.p.) 5 hours after the start of hypothermia. Hypothermic animals were warmed up 48 hours after the inoculation. Also, the increase of the bacterial counts was slower in the hypothermic group, and after as long a time as 40 hours, bacteremia had developed in only 3 of the 8 hypothermic mice, but in all normothermic animals. However, the re-warming was noxious to the hypothermic mice. Three bacteremic animals already in the hypothermic state died during this procedure; two other mice died as well, but without developing any bacteremia; and the remaining three died within 15 hours from the start of the rewarming procedure and proved to be bacteremic when autopsied. Consequently, no significant difference between the two groups as to the mean survival time was found.

A similar experiment is presented in Figure 12. Here, 5,000 Type II pneumococci were given (i.p.) to seven mice five hours after the start of hypothermia which was maintained for 40 hours. Two normothermic control groups were run, one of which was given premedication. The results were similar within both control groups, and they will be treated together.

The results tallied well with those of the previous experiment. Bacterial multiplication was slower in hypothermic mice, and three such mice were protected from bacteremia, whereas all controls became bacteremic. Rewarming was fatal. All surviving mice

PERITONITIS AND BACTEREMIA IN MICE



TUNEVALL AND LINDNER

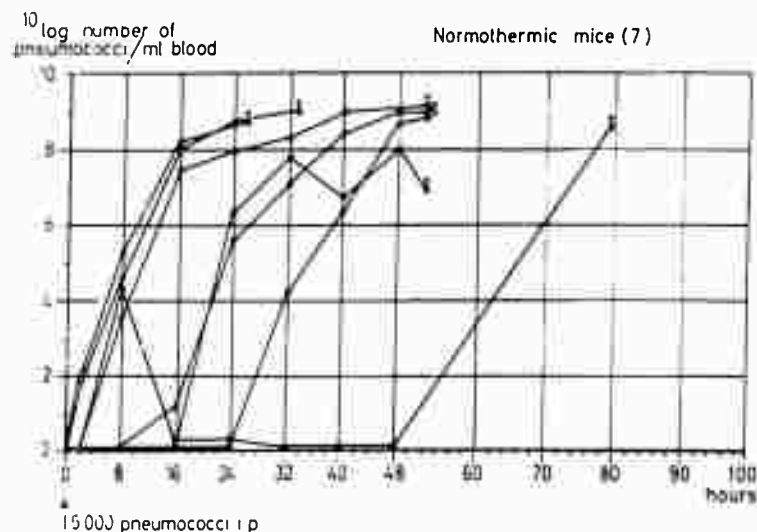


Figure 12. Results of inter vitam and post mortem blood cultures in hypothermic mice and normothermic ones given 200,000 pneumococci Type II intraperitoneally. Markings as in Figure 2.

died; one at start of rewarming, and the remaining two within 12 hours after this procedure. No difference as to the survival time, therefore, was noted.

DISCUSSION

It should be pointed out that our experimental procedure for attaining hypothermia was devised to decrease the body temperature by elimination of the normal thermoregulation at chilling. Thus, stress reactions were minimized and body temperature was rapidly stabilized at 22° C to 23° C.

Preliminary experiments indicated a slower increase of the blood counts of pneumococci and a prolonged survival in hypothermic mice

PERITONITIS AND BACTEREMIA IN MICE

after the induction of pneumococcal peritonitis.

These observations were corroborated by experiments in which bacterial counts from blood were done repeatedly. In other experiments where the infection was less massive the slower course of disease was found to manifest itself in two different ways. First the invasion of pneumococci from the peritoneal cavity to the blood stream was more often delayed in hypothermic animals. If the fairly similar experiments of the Figures 11 and 12 are taken together, 7 out of 15 hypothermic mice were bacteremic 40 hours after the inoculation as against 20 out of 22 controls (Chi-square with Yates' correction 6.9; $0.01 > P > 0.001$). Second, when bacteremia was once established, the increase of the number of bacteria in the blood was slower in hypothermic mice. Our results thus tally with those of Watkyns et al. (1958).

But one other significant observation was made; warming up of hypothermic mice was deleterious. Bacteremic mice died during or soon after this procedure and in mice which still had sterile blood, bacteremia was often established and followed by death within a short time. In others, death occurred without being preceded by bacteremia. It is not possible to decide whether this effect was caused by a weakening of the resistance by hypothermia against bacterial assault as a whole or, more specifically, to an increased sensitivity to bacterial toxins during the rewarming process which in itself is traumatic and may involve an abnormal acceleration of cell metabolism.

SUMMARY AND CONCLUSION

I. Experiments with hypothermic mice maintained at 22° C to 23° C for more than 40 hours, almost invariably resulted in the emergence of bacteria in peritoneal fluid and blood. These bacteria belonged to species present in the intestine. When peritoneal and blood findings were not identical, the demonstration of a species in peritoneal fluid only was more frequent than in blood only. No major

TUNEVALL AND LINDNER

change of the intestinal flora was effected by the hypothermia.

The elimination from the blood stream of injected bacteria was found to be disturbed in hypothermic animals, and even to a greater extent in long periods of hypothermia. *Klebsiellae* rapidly eliminated in normal mice diminished in number only during a period of 2 to 4 hours, but thereafter increased again and caused profuse bacteremia in hypothermic mice.

Treatment with a sulpha-streptomycin combination provoked a shift of the intestinal flora towards a dominance of enterococci and diminished considerably the incidence of bacteria in peritoneal fluid and in blood, but did not influence the survival time of hypothermic mice.

It can be concluded that deep and prolonged hypothermia in mice results in an invasion of bacteria from the intestine into the peritoneal cavity or into the blood stream or both. This invasion is not due to any change of the intestinal flora, but may be contributed to by an inability of hypothermic mice to eliminate bacteria from the blood stream. As antibacterial treatment reduced the incidence of peritonitis and bacteremia but did not increase the survival time of hypothermic mice, the bacterial invasion is not likely to be any important factor for debilitation and death, but rather a concomitant phenomenon.

II. Experimental pneumococcal peritonitis was found not to result in bacteremia as regularly or as early in hypothermic mice as in normothermic controls. Once established, the bacteremia also increased in massivity more slowly in hypothermic mice which also had longer average survival time.

Warming up of the hypothermic mice resulted in a rapidly increasing bacteremia and death, or in death not preceded by bacteremia. This might be due to a diminished resistance as a result of the previous hypothermia, to the trauma inherent in the rewarming procedure, or to an increased susceptibility to bacterial toxins during this process.

PERITONITIS AND BACTEREMIA IN MICE

LITERATURE CITED

I

1. Bennett, L. R., P. E. Rekers, and J. W. Howland. 1951. Influence of infection on the hematological effects and mortality following midlethal roentgen irradiation. *Radiology* 57: 99.
2. Berlin, B. S., C. Johnson, W. D. Hawk, and A. G. Lawrence. 1952. The occurrence of bacteremia and death in cortisone treated mice. *J. Lab. Clin. Med.* 40: 82.
3. Beyer, G. 1956. Die Winterschlafbehandlung in ihrer Wirkung auf die entzündlichen Reaktionen der Gewebe. *Der Chirurg* 27: 275.
4. Billingham, R. E. 1957. Spread of bacteria during hypothermia. *Proc. Roy. Soc. London, Sect. B.* 147: 550.
5. Dienes, L. 1946. Reproductive processes in *Proteus* cultures. *Proc. Soc. Exp. Biol. Med.* 63: 265.
6. Fallowfield, T. L. 1962. The treatment of acutely X-irradiated mice with streptomycin and derivatives of 6-aminopenicillanic acid. *Brit. J. Exp. Path.* 43: 44.
7. Fedor, E. J., B. Fisher, and E. R. Fisher. 1958. Observations concerning bacterial defense mechanism during hypothermia. *Surgery* 43: 807.
8. Fedor, E. J., E. R. Fisher, S. Lee, W. K. Weitzel, and B. Fisher. 1956. Effect of hypothermia upon induced bacteremia. *Proc. Soc. Exp. Biol. Med.* 93: 510.
9. Fischl, E. 1897. Über den Einfluss der Abkühlung auf die Disposition zur Infektion. *Prag. Med. Wochenschr.* 22: 49.
10. Gledhill, A. W., and R. J. W. Rees. 1952. A spontaneous enterococcal disease of mice and its enhancement by cortisone. *Brit. J. Exp. Path.* 33: 183.

TUNEVALL AND LINDNER

11. Gowen, G. F., and G. J. Friou. 1961. The influence of hypothermia on experimental bacteremia in dogs. *Surgery* 50: 919.
12. Hammond, C. W., M. Tompkins, and P. Miller. 1954. Studies on susceptibility to infection following ionizing radiation. I. The time of onset and duration of the endogeneous bacteremia in mice. *J. Exp. Med.* 99: 405.
13. Helmsworth, J., W. Stiles, and W. Elstun. 1955. Leucopenic and trombocytopenic effects of hypothermia in dogs. *Proc. Soc. Exp. Biol. Med.* 90: 474.
14. Laurell, G., and L. Philipson. 1958. Treatment of post-irradiation infection in mice. 3. Studies on the endogeneous bacteremia associated with ionizing radiation. *Acta Path. Microbiol. Scand.* 43: 62.
15. Lindner, T., and G. Tunevall. 1958. Hypothermia and infection. I. Influence of hypothermia on antibody formation in mice in the secondary response to typhoid H-antigen. *Scand. J. Clin. Lab. Invest.* 10: 142.
16. Miller, P., C. Hammond, and M. Tompkins. 1950. The incidence of bacteremia in mice subjected to total body X-irradiation. *Science* 111: 540.
17. Miller, C. P., C. W. Hammond, M. Tompkins, and G. Shorter. 1952. The treatment of post-irradiation infection with antibiotics; an experimental study on mice. *J. Lab. Clin. Med.* 39: 462.
18. Sanders, F., S. Crawford, and M. DeBakey. 1957. Effects of hypothermia on experimental intracutaneous pneumococcal infection in rabbits. *Surg. Forum (Am. Coll. Surg.)* 8: 92.
19. Selye, H. 1951. The influence of STH, ACTH, and cortisone upon resistance to infection. *J. Canad. Med. Ass.* 64: 489.

PERITONITIS AND BACTEREMIA IN MICE

20. Szilágyi, T., L. Koosar, and H. Czernyanszky. 1956. Nervensystem und Immunität. VII. Die Wirkung von Hypothermie auf das Shwartzman-Phänomen. *Acta Microbiol. Acad. Sci. Hungary* 3: 327.
21. Villalobos, T., E. Adelson, and T. Barila. 1955. Hematologic changes in hypothermic dogs. *Proc. Soc. Exp. Biol. Med.* 89:192.

II

1. Balch, H. H., H. E. Noyes, and C. W. Hughes. 1955. The influence of hypothermia on experimental peritonitis. *Surgery* 38: 1036.
2. Bruneau, J., and P. Heinbecker. 1944. Effects of cooling on experimentally infected tissues. *Ann. Surg.* 120: 716.
3. Bürgers, J. 1929. Studien zur Erkältungsproblem. *Schr. Königsberg. Gelehrt. Ges., Nat.-wissensch. Kl.* 6(3): 81.
4. Carpano, M. 1926. Influenza del freddo sul decorso di qualche infezione e sulla virulenza dei relativi agenti patogeni. *Ann. d'Igiene* 36: 787.
5. Grechishkin, D. K. 1956. The influence of artificial hypothermy on the clinical course of experimental sepsis. *Exp. Khirurgiya* 1956 (3): 33.
6. Kligler, I. J. 1927. Relation of temperature to susceptibility of host to disease. *Proc. Soc. Exp. Biol. Med.* 25: 20.
7. Kligler, I. J., and L. Olitzki. 1931. The relation of temperature and humidity to the course of a B. enteritidis infection in white mice. *Am. J. Hyg.* 13: 359.
8. Kligler, I. J., and J. Weitzman. 1926. Susceptibility and resistance to trypanosome infection. II. The relation of physical environment to host susceptibility to infection. *J. Exp. Med.* 44: 409.

TUNEVALL AND LINDNER

9. Lindner, T., and G. Tunevall. 1958. Hypothermia and infection. I. Influence of hypothermia on antibody formation in mice in the secondary response to typhoid-H-antigen. *Scand. J. Clin. Lab. Invest.* 10: 142.
10. Longhin, S., A. Popesco, and D. Volesceanu. 1957. Le rôle de la température dans la généralisation de la syphilis expérimentale. *Arch. Roum. Path. Exp.* 16: 293.
11. Muschenheim, C., D. Dierschner, J. Hardy, and A. Stoll. 1943. Hypothermia in experimental infections. III. The effect of hypothermia on resistance to experimental pneumococcus infection. *J. Infect. Dis.* 72: 187.
12. Previte, J. J., and L. J. Berry. 1962. The effect of environmental temperature on the host-parasite relationship in mice. *J. Infect. Dis.* (in press).
13. Sanders, F., E. S. Crawford, and M. E. DeBakey. 1957. Effects of hypothermia on experimental intracutaneous pneumococcal infection in rabbits. *Surg. Forum* 8: 92.
14. Wotkyns, R. S., H. Hirose, and B. Eiseman. 1958. Prolonged hypothermia in experimental pneumococcal peritonitis. *Surg. Gynec. Obst.* 107: 363.

DISCUSSION

MIYA: How soon after the mouse succumbed did you take your blood cultures?

TUNEVALL: When it occurred in daytime, it was made as soon as we were sure that the mouse was dead. If it occurred during the night, if someone were in the lab, the procedure was the same one. If we were not -- we had to sleep now and then -- a technician would take the mouse directly to the deepfreeze

PERITONITIS AND BACTEREMIA IN MICE

and chill it down there. Then it was cultured the following day.

MIYA: Is it possible that some of your positive blood cultures were just due to the breakdown of your normal tissue barriers before you got the blood?

TUNEVALL: Maybe this could not be entirely excluded, but we have done some model experiments to see if this treatment of the dead mouse very often results in growing through from the intestine, but we have not the impression that this factor has disturbed the results very much.

MARCUS: Yesterday, when the speaker spoke about antibody formation and measurement in rabbits, he spoke about it in rabbits using quantitative antibody estimations by classical methods of determination. He did not consider the possibility of formation of non-precipitating antibody, and I think that this is probably valid because most rabbits don't ordinarily form too much of this. But you are using mice, and I wondered if this might not be a significant consideration. I don't know what the system was that you employed for measuring the antibody, but I wondered if you had given it any consideration at all?

TUNEVALL: We used the typhoid-H antigen, and for determining the antibody level, we simply used the Widal test.

MARCUS: One other thing I wanted to ask about. In discussing the mechanism of invasion of the hypothermic mouse, in comparing it to what occurs in the irradiated animal, there are some interesting possibilities. As far as I know, no one knows the molecular lesions that occur following irradiation which lead to invasion, but there is good evidence at the cellular level of what the lesions and resistance are. The mouse does not have any bactericidal component in its serum, but following irradiation there is not a loss of phagocytic capacity, but a loss of intracellular destructive capacity, and I wonder if this is the same type of lesion which might occur in the hypothermic mouse.

TUNEVALL: I'm sorry, I cannot answer that question.

TUNEVALL AND LINDNER

MARROW: I will describe two clinical observations. An infant with considerable cold exposure, due to drunkenness of the parents, was brought into the hospital, failed to respond to massive antibiotic therapy, and the youngster died with peritonitis, which would be exactly parallel to your experiments taken over into a human, although the degree of lowering of body temperature, we do not know. The parents were inebriated to the point of anesthesia for a period of a couple of days, so we don't know how long the youngster was hypothermic. We have had six hypothermic, or, let's say, "sub-normal temperature" individuals below the age of three months. The first two were treated conventionally with massive doses of penicillin, both dying within the third or fourth day of hospitalization. The next four were given massive doses of gamma globulin immediately upon admission, and had relatively uneventful recovery.

THE ROLE OF LOW ENVIRONMENTAL TEMPERATURES IN PREDISPOSING MICE TO SECONDARY BACTERIAL INFECTION¹

Gennaro J. Miraglia² and L. Joe Berry

ABSTRACT

The LD₅₀ for mice of *S. typhimurium*, strain RIA, is 4.1×10^5 cells for animals individually housed without bedding and maintained at 25° C. It is 3.8×10^3 cells for animals similarly housed but kept at 5° C. However, mice are able to withstand nearly 100 times this dosage of strain RIA if they are housed in groups at 5° C. Normal mice with their dorsal and ventral surfaces shaved are unable to survive more than a few days when housed individually in the cold, but survive beyond two weeks under group housing conditions. No effect of cold could be detected in mice infected with the highly virulent SR-11 strain of *S. typhimurium* since all animals died following infection with only a few cells. Mice that were natural carriers of salmonellae as judged by fecal discharge were highly resistant to challenge and responded to cold in a manner similar to normal mice infected with RIA. Strain RIA could be isolated from the tissues of infected animals with greater frequency and persisted longer in mice maintained at 5° C than those at 25° C. Coagulase negative staphylococci were isolated from liver, lung, spleen, heart, and kidney of animals that survived salmonellosis for 14 days at 5° C. The staphylococci did not appear to have a predilection for one tissue over another, and were isolated in an incidence proportional to the number of salmonellae injected in the primary infection. At 25° C, only a small percentage of mice had staphylococci in tissues, and these occurred independent of the infectious dose of salmonellae. These observations were made on normal mice infected with RIA and on carrier mice infected with SR-11. The origin of the secondary invader remains obscure, but it does not appear to result from a penetration of coagulase negative staphylococci from the lumen of the gut to the deep tissue. Mice devoid of intestinal staphylococci and recolonized with coagulase positive staphylococci continue to show coagulase negative isolates from deep tissue.

¹ This work was supported in part at Bryn Mawr College by Contract AF 41(657)-340 between Bryn Mawr College and the Arctic Aeromedical Laboratory, USAF, and in part by Training Grant 2E-148, U. S. Public Health Service.

² Postdoctoral Fellow on Training Grant 2E-148. Present address: Department of Microbiology, Seton Hall College of Medicine, Jersey City, New Jersey.

MIRAGLIA AND BERRY

The extent to which exposure of experimental animals to low environmental temperature alters or modifies the outcome of host-parasite interaction is not clearly established. Pasteur, nearly a century ago, attributed the resistance of chickens to anthrax to the characteristically high body temperature of fowls. By inducing hypothermia he was able to render them lethally susceptible. In the intervening years, reports dealing with the effects of cold on the course of bacterial infections have been few in number. On the other hand, literature concerned with the physiological effects of exposure to low temperatures on various mammalian species is extensive, including several reviews (Hemingway, 1945; Hardy, 1950; Hardy, 1961; and Smith and Hoijer, 1962).

There are, nevertheless, important studies on the contribution of the environment to the response of animals challenged with several different infectious agents. Junge and Rosenthal (1948), for example, studied the survival of mice infected with pneumococci and reported increased susceptibility when the temperature was decreased to 18° C. It was necessary, however, to treat the animals with sulfadiazine immediately following infection in order to insure sufficient survival to make the temperature effect apparent.

Muschenheim and collaborators (1943) had studied several years earlier the effect of hypothermia in rabbits on resistance to experimental pneumococcal infection. When a highly virulent strain was employed, the only demonstrable effect of lowered body temperature on host response to the pathogen, compared to that in normothermic animals, was a decrease in the local inflammatory reaction. When a strain of low virulence was used, the induced hypothermia resulted in bacteremia and death in addition to the inhibition of the dermal inflammatory reaction.

The interaction between certain viruses and a variety of hosts as influenced by environmental temperature has received considerable attention in recent years. The incisive investigations of Boring et al. (1956) are particularly germane to this paper. Cold was found to have an adverse effect on the mouse infected with a Cosackie virus. The animals were housed 8 to 12 per cage at 4° C without restriction on huddling. At this temperature there was a viremia through the fourth post-infection day while the blood was clear of virus by that

SECONDARY BACTERIAL INFECTION IN MICE

time in mice at 25° C. Similarly, the titer of virus in the liver was higher on the fourth day in mice at 4° C than in mice at 25° C. These experiments indicate that although adult mice possess a natural resistance to the Coxsackie virus such that the disease is limited to a non-fatal infection, this resistance is lost when animals are maintained in the cold. Under these conditions, a lethal infection ensues which is characterized by a persisting viremia, high levels of virus in the liver, and lesions demonstrable in other organs. The mechanism by which cold reduces resistance to the virus is unknown, although the fact that cortisone causes a similar loss of resistance suggests that cold may act through its capacity to augment secretion of adrenocortical steroids. It may also result in the involvement of other body responses less well defined. Thus, Walker and Boring (1958) observed that neutralizing antibody appeared on the fourth day in mice at room temperature but failed to appear in animals in the cold. Injections of cortisone are known to suppress the immune response (Germuth, 1956).

Schmidt and Rasmussen (1960) reported that mice maintained at 37° C were more resistant to infection with herpes simplex virus than those held at 25° C. This protective effect was believed to be due to the lower viral population in brain tissue at the higher temperature. The mechanism responsible for this decrease in the number of viruses is unknown, but a possible explanation for the difference in mortality rates is an alteration in viral multiplication due to a temperature induced change in the metabolism of host tissues. It has been well established that viral populations can be controlled to some extent by only a few degrees change in temperature (Lwoff, 1959).

The object of the present study was to determine possible differences in the course of salmonellosis in mice maintained at 25° C with others kept at 5° C, and to uncover, if possible, mechanisms responsible for such differences. It was not our intention, however, to employ mice with a controlled hypothermia. In the first place, the lower environmental temperature to which animals were subjected failed to depress the core temperature below the normal range. In addition, to attempt to regulate the degree of hypothermia in populations of mice the size of those used in the experiments would have been technically beyond the resources available. The ability of mice

MIRAGLIA AND BERRY

to maintain normothermia when kept at 5° C for 10 to 15 days has been reported previously by Bischoff (1942). Nevertheless, moribund animals in the cold do become markedly hypothermic, as they do during the last hours of life at room temperature when suffering from salmonellosis.

The data to be presented confirm and extend those of Previte and Berry (1962) who demonstrated an increase in host susceptibility to infection with Salmonella typhimurium when a strain of low virulence but not one of high virulence was used in mice continuously exposed to a low ambient temperature. In addition, and perhaps of even greater importance, is the observation that a second invading organism, a coagulase-negative staphylococcus, presumably from the environment or from the mouse itself, becomes established in tissues of the host already stressed by cold and the experimental salmonellosis. It has long been believed that primary infections predispose an animal to secondary invasion but, heretofore, experimental evidence for this has not been clear. To the authors' knowledge, this is the first description of a situation which permits consistent prediction of the incidence of secondary infection.

MATERIALS AND METHODS

Animals. CF₁ female mice (Carworth Farms, New York City, New York) weighing 20 to 22 gm were used in all experiments. They were housed in plexiglass cages which were divided into 10 equal compartments per cage. Each compartment measured 3.5 x 7.5 x 9.0 cm. No bedding was used, and mice were housed individually to prevent huddling. The open tops and bottoms of the plexiglass enclosures were covered with 3/8 inch mesh hardware cloth. All completely assembled cages were placed on wire mesh to keep the animals free from excessive moisture and excreta. Water and pathogen-free mouse food (Dietrich and Gambrill, Frederick, Maryland) were available at all times.

Animal Rooms. Two animal rooms were used; one was maintained

SECONDARY BACTERIAL INFECTION IN MICE

at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, the other at $5^{\circ}\text{C} \pm 1^{\circ}\text{C}$. An automatic lighting system provided 12 hours of light for each 24 hour period. The mice were kept continuously in the appropriate room for the entire experimental period. Humidity was not controlled but results were reproducible at different seasons of the year when humidity is known to vary.

Inoculum. Two strains of *Salmonella typhimurium* were used, the highly virulent SR-11 and the relatively avirulent RIA. Both strains were cultivated in brain-heart infusion broth (Difco) for 16 hours at 37°C . Decimal dilutions of the cultures were prepared with pyrogen-free saline (Baxter Laboratories, Morton Grove, Illinois) and were plated in triplicate on both nutrient and SS agar plates (Difco) to enumerate the colonies and to insure the uniformity of the culture. Inoculations consisting of the appropriate number of organisms contained in 0.5 ml of saline were administered by the intraperitoneal route.

Organ culture technique. Tissues (heart, lung, kidney, spleen and liver) selected for bacterial culture were excised by aseptic technique from mice immediately after death. Samples were cultured on appropriate media for identification of microflora, using the organ print method. Nasal cultures were also desired, but owing to the obvious difficulties in attempting to obtain inocula from the nasopharynx, only the external nares of the animals were cultured by the imprint method on appropriate medium for staphylococci.

Stool culture technique. Stool cultures were made, where indicated, by placing into the appropriate medium a single fecal pellet obtained on a sterile swab at the time of defecation. To culture for staphylococci, a pellet was incubated at 37°C in brain-heart infusion broth containing 7 per cent sodium chloride for 16 to 18 hours before streaking on *Staphylococcus* 110 medium (Difco). To culture for salmonellae, a second specimen was similarly incubated in selenite broth (Difco) before streaking on SS agar.

Coagulase test. The coagulase test was conducted in Wassermann tubes using 0.5 ml of reconstituted coagulase plasma (Difco) to which was added two drops of a 16 hour brain-heart infusion culture of the staphylococcus under test. Tubes were read after three hours incu-

MIRAGLIA AND BERRY

bation at 37° C.

Miscellaneous. In lieu of drinking water, 0.01 N hydrochloric acid (pH 2.0) was given to mice to rid the gut of staphylococci. This is a modification of the method of Schaedler and Dubos (1962). The absence of the organisms was confirmed by stool culture. Where recolonization of the intestine with a specific strain (in this case Staphylococcus aureus, strain Giorgio) was desired, the hydrochloric acid treatment was terminated before feeding the mouse for a period of 12 hours the desired microorganism as a contaminant in the ration.

RESULTS

Determination of LD₅₀. The LD₅₀ for animals infected with strain RIA and maintained at 5° C and 25° C was determined by the method of Reed and Muench (1938). This was found to be 4.1×10^5 cells per mouse at 25° C and 3.8×10^3 cells per mouse at 5° C, as shown in Table I. The LD₅₀ for the highly virulent SR-11 strain was less than seven cells per mouse at room temperature, hence a temperature effect was not demonstrable. All observations were terminated after a period of 14 days.

The space limitation imposed on mice housed in the compartmented cages did not alter the LD₅₀ dose of the RIA strain of S. typhimurium at 25° C. This was true also for the LD₅₀ dose of crude bacterial endotoxin administered by intraperitoneal injection. Neither crowding nor the psychological effects of isolation under a situation where neighboring mice were visible through the clear plexiglass made any measurable difference in these animals compared to those normally housed.

The same type of control experiment could not be conducted, however, at 5° C since animals permitted to huddle are not as severely stressed by cold as those kept in isolation. This can be seen from the following experiment. Animals were housed 10 per cage (10 x 7 x 6 1/2 inches) with pine shavings as bedding and without restriction

SECONDARY BACTERIAL INFECTION IN MICE

Number of mice dead/total tested in mice group housed at:

5° C		25° C	
6/10	60%	17/30	56.6%

Table I. The LD₅₀ dose of *S. typhimurium* for mice as influenced by bacterial strain and environmental temperatures.

Inoculum	Temperature	LD ₅₀
Strain RIA	25° C	4.1×10^5
Strain RIA	5° C	3.8×10^3
Strain SR-11	25° C	7 cells
Strain SR-11	5° C	7 cells

Table II. Effect of group housing on survival of mice at 5° C and 25° C infected with 4.8×10^5 cells of *S. typhimurium*, strain RIA. Single housing, LD₅₀ = 3.8×10^3 RIA at 5° C. Single housing, LD₅₀ = 4.1×10^5 RIA at 25° C.

on huddling or activity. They were inoculated with 4.8×10^5 cells of strain RIA and for those maintained at 25° C, 17 out of 30 (56.6 per cent) died (Table II). This was the anticipated result since this inoculum is the approximate LD₅₀ for mice at room temperature. On the other hand, mice similarly housed and inoculated and placed at 5° C showed six out of 10 deaths (60 per cent) in response to an inoculum that is characteristically 100 times the LD₅₀ for animals housed individually in the cold. From these findings it is apparent that cold modifies the response of mice to infectious challenge only under specific conditions of exposure. This is important to keep in mind in comparing the experiments described here with those reported elsewhere.

In another experiment utilizing 20 normal mice, 10 were shaved on their dorsal and ventral surfaces and housed individually; the remaining 10 were also shaved but were group housed with pine shavings as bedding. In 24 hours, eight of the 10 mice housed individually died and all were dead by the fifth day. The group housed mice, however, lived for the entire 14 day experimental period and were then sacrificed. This points up once again the importance of housing conditions in experiments on cold.

MIRAGLIA AND BERRY

Number of *S. typhimurium* injected intraperitoneally into mice kept the temperatures indicated

Days Post Infection	4.8×10^2		4.8×10^3		4.8×10^4		4.8×10^5	
	25°	5°	25°	5°	25°	5°	25°	5°
1								
2					1			
3					1		2	
4							4	
5		1			1		3	5
6			2				5	2
7		3	3		1		1	3
8	1		1		2		3	
9			1		1			
10			2		2			
11		1	1		1			
12					1			
13					1			
14								
Dead Tested	$\frac{1}{10}$	$\frac{5}{10}$	$\frac{0}{30}$	$\frac{11}{30}$	$\frac{0}{30}$	$\frac{12}{20}$	$\frac{9}{20}$	$\frac{19}{20}$

Table III. The effect of temperature on the distribution of deaths with time in mice infected with graded doses of *S. typhimurium*, strain RIA.

SECONDARY BACTERIAL INFECTION IN MICE

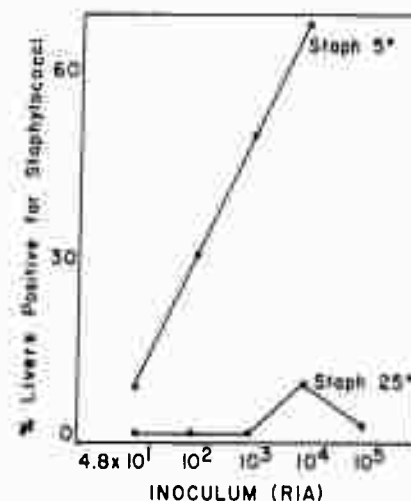


Figure 1. The per cent of liver cultures positive for staphylococci in mice at 5° C and at 25° C as related to the infecting dose of *S. typhimurium*, strain RIA. All liver cultures were made 14 days postinfection.

In order to test the possibility that a cold environment enhanced the virulence of the organism (strain RIA), isolates were recovered from the liver and spleen of infected mice sacrificed for the purpose after being held at 5° C for various periods up to 14 days. These isolates were injected into normal mice and the LD₅₀ and mean survival times were determined at both temperatures. No differences from the parent strain were noted, nor were there any detectable changes in colonial morphology or growth rate in vitro when compared to the original strain. Using these criteria, it would appear that a cold environment does not alter under in vivo conditions the virulence of the invading organism.

Table III shows the mortality rates of mice at room temperature and in the cold when given graded doses of strain RIA. It is apparent that animals maintained in the cold are not only more susceptible to infection, as judged by the increased mortality rate, but the initial deaths occur sooner than those at 25° C. When mice are injected with an LD₅₀ dose of strain RIA (4.8×10^5 cells per mouse at 25° C, as determined directly and not by calculation, as mentioned above),

MIRAGLIA AND BERRY

a highly significant difference is noted in the mortality ratios between the two temperatures. This difference is significant at the 0.8 per cent level by rank test (White, 1952).

Influence of temperature on the per cent of livers positive for bacteria. At the termination of each experiment, all animals that survived the 14 day period of observation were killed by cervical dislocation. The livers were immediately excised and cultured on nutrient agar, MacConkey's agar, SS agar, and Staphylococcus 110 agar by the print method. The results are shown in Figure 1 for the experiments conducted with strain RIA. Not indicated on this figure was the observation that the per cent of mice from which salmonellae could be isolated was greater in animals kept in the cold than in those at 25° C. Moreover, as might be expected, the per cent of positive livers increased in proportion to the size of the infectious dose. Even more important was the number of mice which gave staphylococci from liver imprint cultures. This secondary invader, as can be seen from Figure 1, is to be found in animals kept at 5° C in an incidence that is proportional to the number of salmonellae (RIA) administered in the primary infection. For mice housed at 25° C, a proportionality between primary and secondary infection is not at all apparent. It would seem, therefore, that a combination of cold and salmonellosis predisposes to an invasion of staphylococci. When inocula exceed the LD₅₀ dose for mice at 5° C, few mice survive to be tested for tissue staphylococci and hence an atypical group, the highly resistant animals, survives. Thus, the relationship between primary and secondary infection was unpredictable, as indicated by the curves plotted for the 10⁴ and 10⁵ inocula. Despite the tendency toward selection of atypical survivors, the data obtained with livers cultured for staphylococci continued to show a substantially higher incidence of secondary invasion for mice in the cold than for animals at room temperature.

The effect of low temperature on salmonella-carrier mice. Results similar to those obtained with strain RIA were noted when the highly virulent strain SR-11 was used in experiments with mice which proved to be typhoid carriers. The results of this study, using mice which arrived from the dealer with feces that yielded positive cultures for salmonellae, are shown in Figure 2. Thus, normal mice infected with avirulent salmonellae and exposed to cold show

SECONDARY BACTERIAL INFECTION IN MICE

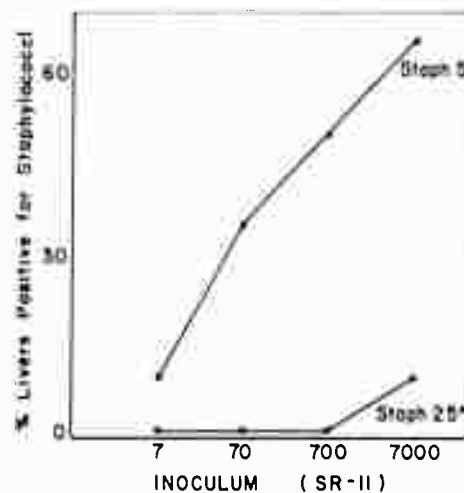


Figure 2. The per cent of liver cultures positive for staphylococci in natural salmonella carrier mice at 5° C and at 25° C as related to the infecting dose of *S. typhimurium*, strain SR-11. All liver cultures were made 14 days postinfection.

an incidence of livers positive for staphylococci in the same way as do carrier mice in the cold infected with virulent SR-11. Staphylococci were not present in livers cultured from carrier mice maintained at 25° C except those infected with the highest dose of strain SR-11, and even here only 10 per cent of the mice had a positive culture. The high degree of immunity of the carrier mice is evidenced by the fact that all animals at room temperature were able to withstand 7000 LD₁₀₀ of strain SR-11, while only four of 10 animals died from this dose at 5° C. The SR-11 strain is usually fatal to mice when only a single cell is injected (Schneider and Zinder, 1956). Even with enhanced resistance, some carrier animals succumbed at 5° C. The death of these mice shows that exposure to low environmental temperature increases susceptibility to infection even in highly immune mice.

It might be suggested that as increasing numbers of salmonellae are injected, progressively mounting stress is applied to a host already stressed by cold so that staphylococci already in the host or its environment now become established in its tissues. Noteworthy

MIRAGLIA AND BERRY

also was the observation that from a group of 30 non-infected controls held at 5° C for 14 days, only 23.3 per cent had liver cultures positive for staphylococci and from none could salmonellae be obtained. A similar number of control mice at 25° C had negative liver cultures for both salmonellae and staphylococci. This further supports the contention that animals in the cold have a decreased capacity to resist infection.

Efforts have been made to determine the origin of the staphylococci which appear in the tissues of cold exposed mice. Most of the normal animals (95 per cent or 294 of 308) discharge staphylococci in the feces, but only 1 per cent approximately are coagulase-positive strains. These figures remain essentially unaltered in normal mice exposed to cold. Since coagulase-negative strains do not lend themselves readily to phage typing, a correlation between the staphylococci isolated from tissues and those found in feces of the same mouse could not, therefore, be attempted.

An indirect method was employed, however, using a modification of the technique of Schaedler and Dubos (1962) which consists of substituting 0.01 N hydrochloric acid for the drinking water in order to rid the gut of staphylococci.

Animals have been maintained on hydrochloric acid drinking water for over 40 days without any obvious untoward effects. The appearance and behavior of the animals was normal. There was normal weight gain and growth, and the pH and character of the stools was indistinguishable from that of normal controls.

Effect of acid water treatment on the per cent of mice with feces positive for staphylococci. In most experiments five to seven days of acid treatment sufficed to rid the gut completely of culturable staphylococci. In this connection, however, a seasonal effect was noted in experiments conducted during the summer months even though the animals had air-conditioned quarters. At this time of year, additional time on acid treatment was required to free the intestine of staphylococci, as shown in Table IV. Moreover, while studies conducted during the winter consistently yielded coagulase-negative staphylococci from the feces, summer studies revealed a low percentage of coagulase-positive strains as well as a high

SECONDARY BACTERIAL INFECTION IN MICE

Duration of HCL treatment (Days)	Per cent of mice with fecal staphylococci	Per cent coagulase negative	Per cent coagulase positive
0	95 (308)	95	0
6	67 (15)	67	0
10	11 (27)	11	0
12	5 (20)	5	0
28	40 (20)	30	10

Table IV. Staphylococci recovered from the feces of mice after various periods of hydrochloric acid treatment. Number of mice tested shown in parentheses.

percentage of coagulase-negative strains. When no staphylococci could be cultured from the feces after a period of acid treatment, the digestive tract was then recolonized with strain Giorgio, a coagulase-positive staphylococcus, and for about a week thereafter the stool cultures contained only coagulase-positive strains. While mice continued to shed these strains for at least several additional weeks, they also began to discharge coagulase-negative strains by the 14th day. This occurred in animals maintained at 25° C and 5° C.

Although the gut can be made free of staphylococci by treatment with hydrochloric acid, these organisms became reestablished in approximately 50 per cent of the animals within several days after tap water was substituted for the acid. In fact, in animals autopsied after the usual experimental period of 14 days, coagulase-negative staphylococci were found in nearly all stool cultures. This too was independent of the environmental temperature at which the mice were kept.

Effect of acid water treatment on the per cent of mice with tissues positive for staphylococci. Deep tissue invasion has, with only a few exceptions, consisted of coagulase-negative

MIRAGLIA AND BERRY

Treatment	Per cent of tissues positive for staphylococci*	
1 day at 5° C	0	(5)
5 days at 5° C	0	(5)
8 days at 5° C	40	(5)
14 days at 5° C	50	(18)
21 days at 5° C	40	(5)
14 days at 25° C	0	(10)
10 days HCl + 5 days at 5° C	33	(3)
10 days HCl + 14 days at 5° C	56	(9)
10 days HCl + 14 days at 25° C	10	(10)

Table V. Staphylococci recovered from tissues of mice after various treatments. All isolates were coagulase-negative. Number of mice tested shown in parentheses. *Tissues tested: liver, kidney, spleen, lung and heart.

strains. Table V summarizes the results. The intestine when colonized with the coagulase-positive Giorgio strain could not be the origin, therefore, of the secondary invader. But even more revealing is the fact that staphylococci were still isolated from the tissues of cold exposed mice with the anticipated frequency in individuals whose feces contained no culturable staphylococci.

Reestablishment of coagulase-negative staphylococci in the gut of mice rendered free of staphylococci by acid treatment did not alter the frequency of tissue invasion by coagulase-negative organisms. This too is evidence against the possibility that the invaders are intestinal in origin.

In tissues of acid-treated cold exposed mice, a few cases were recorded in which both coagulase-positive and coagulase-negative strains were found in the same individual but only one type was present in any particular organ. In cases where coagulase-positive

SECONDARY BACTERIAL INFECTION IN MICE

Duration of HCl treatment (Days)	Per cent of mice with nasal staphylococci		Per cent coagulase negative	Per cent coagulase positive
0	100	(60)	100	0
10*	100	(78)	95	5
12	100	(80)	100	0
28*	100	(10)	90	10

Table VI. Staphylococci recovered from the noses of mice after various periods of hydrochloric acid treatment. All mice were nasal carriers of staphylococci before acid treatment. Number of mice tested shown in parentheses. *Summer mice.

staphylococci were isolated from the tissue, the nasal flora likewise consisted of coagulase-positive strains. These data, in conjunction with those above, implicate the respiratory tract (nares) as a possible focus from which secondary invaders arise.

The data of Table V demonstrate that secondary invasion by staphylococci in normal mice exposed to cold requires approximately a week and appears to reach a maximum in 14 days. Prior treatment of the mice with hydrochloric acid drinking water did not alter significantly, moreover, either the incidence or timing of staphylococcal involvement of deep tissue.

In view of the above, and since it is well known (Taylor and Dyrenforth, 1938) that acute cold adversely affects the upper nasal passages, this area was studied to determine if it might serve as a possible portal for deep tissue invasion.

Effect of acid water treatment on the per cent of mice with noses positive for staphylococci. Table VI shows that the percentage of mice with culturable staphylococci from the nose remains essentially unaltered regardless of the experimental procedures to which the animals are subjected. For example, hydrochloric acid drinking water given for various periods up through 28 days did not lower the per cent of staphylococcal nasal carriers among normal or Giorgio fed mice. This is contrary to what was noted in the gut since it could be freed of staphylococci following acid water treatment and then re-

MIRAGLIA AND BERRY

colonized with the Giorgio strain with great facility. Thus, nasal staphylococci which are mainly coagulase-negative are found to persist unabated in all 228 mice studied.

Nasal cultures also revealed that in the winter only coagulase-negative strains were harbored, but that in the summer a low incidence of positive strains was also evident. In a group of mice from which coagulase-positive strains were isolated from the tissues for the first time, it was found that the stools contained only coagulase-negative staphylococci, whereas also for the first time coagulase-positive organisms were isolated from the nose. Coagulase-positive strains have never been isolated from the tissues of mice which had coagulase-negative nasal flora. Thus, a correlation may exist between the nasal flora and the organisms isolated from deep tissue as secondary invaders in cold stressed mice.

Effect of acid water treatment on the per cent of salmonella carrier mice with feces positive for salmonella. It is reasonable to assume that since hydrochloric acid treatment eradicates staphylococci from the gut, the population of other members of the intestinal microflora may be likewise altered. This phase of the study has not been actively pursued, but results from a preliminary experiment using 40 mice show that the carrier rate for salmonella was reduced from 80 per cent to 5 per cent, $P < .008$ (Wilcoxon, 1949), in 24 hours by using the acid water treatment. This indeed appears to be a dramatic reduction, but owing to the inherent shortcomings of the sampling method and the obvious danger of generalizations based on a single experiment, a more definite statement concerning the efficacy of this treatment for carrier mice must await more intensive studies.

DISCUSSION

Host defenses are breached in animals maintained in the cold. The two levels of cellular defense, one comprised of the more peripheral wandering phagocytes and the other the deeper fixed tissues of the reticuloendothelial system (RES), are each affected by cold or

SECONDARY BACTERIAL INFECTION IN MICE

hypothermia. Halpern et al. (1951) studied the activity of the RES, as judged by its ability to clear colloidal carbon, in hypothermic rats and found a decided reduction in function. In rats at normal temperatures, 90 per cent of the carbon was "fixed" in the RES in 35 minutes, while in the hypothermic animal 29 per cent was sequestered.

Frohlich (1938), in studies of wandering phagocytes, found an increase in number of polymorphonuclear leucocytes in hypothermic rabbits, as noted by others, but up to 65 per cent of the cells were either injured or were atypical. Similarly, Taylor and Dyrenforth (1938) reported an impairment of phagocytic activity of fixed tissue cells in human subjects immersed in water at 20.3° C. It was claimed, moreover, that low environmental temperatures predisposed to infections, especially in the upper respiratory region, but the evidence was not convincing, primarily on the basis of sample size. A decrease in blood content of complement and opsonin was found by Wildfuhr (1950) in persons exposed to cold. Thus, the humoral as well as the cellular defense is said to be altered by cold. In some host-parasite systems, therefore, low ambient temperatures are generally deleterious and seem to enhance not only an infection already underway but seem to "unmask" any secondary involvement.

Attempts to compare data obtained from various laboratories suffer, unfortunately, from the lack of adequate standardization in experimental design. That different host-parasite models are used assumes little importance in face of the realization that not all investigators report the duration of the photo period per day and the housing conditions employed. Furthermore, the term "cold", depending on the investigator, frequently spans sizeable temperature ranges. Even the conditions used in the studies just described are quite artificial and may not have a counterpart in nature. Animals were subjected to a constant and unfluctuating cold. This forces them to live at a level of high energy expenditure for long periods, a condition seldom known to occur with any certainty in nature. Moreover, the photo period was always 12 hours of light per day, and the light intensity was constant. This, too, of course, is contrary to the natural state. In spite of these apparent shortcomings, the results were constant and reproducible. Evidence for a decreased host resistance in the cold to infection with *S. typhimurium* is convincing and is even easier to accept in view of the increased incidence of

MIRAGLIA AND BERRY

secondary infection with staphylococci in mice maintained in the cold.

The differences in host behavior at room temperature and at low temperature in response to salmonella infection is more apparent at infectious dosages below or at the approximate LD₅₀ level. With heavier inocula, homeostatic balance becomes erratic. This is especially true in experiments in which attempts were made to culture organs for bacteria at the 14th post-infection day. In this regard, data not included in this report indicate that while the per cent of salmonella positive livers is greater in animals held at 5° C than in those at room temperature, this difference becomes less pronounced as heavier inocula are employed. The per cent of livers positive for staphylococci, however, increase with increasing dosages of salmonellae, especially of strain RIA.

There still remain to be answered several perplexing questions. Not the least of these is the observation that while by their very nature staphylococcal infections tend to localize and form well-defined foci of infection, this has never been observed in the hundreds of animals autopsied during the course of these investigations. Gross examination of the nasal passages and sinuses failed to show a localized pathology in 14 days, the usual term of these studies. The invasion involves lung, heart, kidney, spleen and liver in an unpredictable manner and without any apparent preference for any specific tissues, this in face of the usual course of events in which the staphylococci frequently invade the kidneys with subsequent overt signs.

Paradoxical also has been the observation that while mice fed the coagulase-positive Staphylococcus aureus, strain Giorgio, as a contaminant in their ration following acid treatment become intestinal carriers of this strain, they also become nasal carriers of the same strain, perhaps by the manner in which they eat. This state rarely lasts for more than 48 hours nor are more than 30 to 40 per cent of the mice such transient carriers. Even so, these individuals never yielded coagulase-positive isolates from deep tissue.

It would appear, then, that while a correlation seems to exist between the flora of the nose and that of deep tissue, this can not, under

SECONDARY BACTERIAL INFECTION IN MICE

the conditions of these experiments, be altered experimentally so that a coagulase-positive strain established artificially in the host by eating infected food be subsequently made to invade deep tissue.

The manner in which infectious agents reach potential victims, enter them and establish themselves with subsequent detriment to the host has been recognized since the "Golden Era of Bacteriology". What needs further elucidation are the mechanisms responsible for the absence of overt disease symptoms in hosts parasitized by virulent pathogens which are known to persist for long periods of time.

The microorganism possessing the weapons of infectivity and pathogenicity upon entrance into a suitable host need not cause disease. This bespeaks of the complexity of the host-parasite relationship. Those studying the infectious process have long been aware that many "normal" animals harbor in their tissues a variety of parasites including viruses and bacteria. There are reports in the literature of a high incidence of the virus of polio and herpes simplex and the microbe of tuberculosis, indicating that the ability of the animal to remain free of clinical signs despite invasion exceeds its ability to prevent microbial and viral penetration. Thus, it may not be surprising that staphylococci are found in deep tissues of mice, but why its incidence is increased when the host is stressed by cold or cold and primary infection requires answer.

There is every reason to believe that there are a number of agents that may parasitize man without his knowledge and are exacerbated only during periods of stress. There are, for example, reports suggesting that herpes simplex expresses its clinical picture during physical and emotional disturbances. Likewise, clinical tuberculosis is manifested in patients stressed by poor nutrition or debilitated by another disease (primary infection). Thus, pathogens or potential pathogens can and in some instances do persist without clinical symptoms. Detection of these elusive agents depends upon adequate procedures and, therefore, come to light only after requisite advances in technology. Recovery of salmonellae from the excreta of individuals with typhoid fever, or from the blood and other tissues during certain stages of the infection, can be accomplished successfully with present bacteriological methods. However, when the host

MIRAGLIA AND BERRY

is a carrier (asymptomatic), attempts to culture the organism become more difficult. This aspect of the problem is not unique for analogies exist in other systems. In rodents, the etiological agent of pseudotuberculosis cannot be isolated from the animal in the normal state, but can be induced to multiply rapidly in individuals under stress or those given large doses of cortisone.

It is reasonable to assume that the nature of the infected tissue itself may contribute to the difficulty attending efforts to uncover the presence of pathogens. Tissue fluids containing either antibodies or other inhibitors transferred along with the pathogen may prevent its ultimate detection not because of its absence but because its multiplication on suitable substrate is prevented. It becomes necessary under these conditions to remove the effect of the inhibitory substances either by simple dilution or by more sophisticated procedures before the pathogen can be successfully demonstrated. Since uncovering procedures are implemented only with considerable difficulty, a more indirect approach might be utilized in attempts at "unmasking"; that is, by stressing the host to a level where its influence on the parasite becomes minimized. An explosive replication of the pathogen would then permit its presence to be detected by standard procedures. A stratagem of this type successfully executed would do much to broaden our knowledge of agents whose presence otherwise escapes us.

In recent years, virologists have provided additional evidence that latent viral infections are common to man as well as animals. Data are also accumulating which suggest that latent infections of bacterial etiology may be equally common. Approximately half of the normal population harbor in their nasopharynx coagulase-positive staphylococci, and undoubtedly other agents will be detected when sought with greater effort.

That such infections exist and are capable under the proper conditions of causing overt disease more than justifies any attempts at applying in carefully controlled experiments stressors which will assist in their detection.

SECONDARY BACTERIAL INFECTION IN MICE

SUMMARY

1. The LD_{50} dose for mice of S. typhimurium, strain RIA, is 4.1×10^5 cells per mouse for animals individually housed without bedding and maintained at $25^{\circ}C$. It is 3.8×10^3 cells per mouse for animals similarly housed but kept at $5^{\circ}C$.

2. No effect of cold could be detected in mice infected with the highly virulent SR-11 strain of S. typhimurium since all animals died following infection with only a few cells. Mice that were natural carriers of salmonellae as judged by fecal discharge were highly resistant to challenge and responded to cold in a manner similar to normal mice infected with RIA.

3. Strain RIA could be isolated from the tissues of infected animals with greater frequency and persisted longer in mice maintained at $5^{\circ}C$ than those at $25^{\circ}C$.

4. Staphylococci were isolated from livers of animals that survived salmonella infection for 14 days at $5^{\circ}C$ and the incidence of staphylococci was proportional to the number of salmonellae injected. At $25^{\circ}C$, only a small percentage of mice had staphylococci in tissues and these occurred independent of the infectious dose of salmonellae. These observations were made on normal mice infected with RIA and on carrier mice infected with SR-11.

5. The feeding of 0.01 N hydrochloric acid to mice in lieu of drinking water is apparently harmless to the general well-being of the animals under the conditions indicated, but rids the gut of all culturable staphylococci in five to seven days in experiments conducted in the winter. The period of hydrochloric acid treatment must be extended to achieve comparable results in summer studies. Neither ridding the gut of the normally present coagulase-negative staphylococci nor establishing a coagulase-positive strain by the feeding of contaminated food altered the incidence of tissue invasion by coagulase-negative organisms.

6. Hydrochloric acid treatment failed to alter the incidence of

MIRAGLIA AND BERRY

nasal staphylococcal carriers. Hence, the origin of the secondary invading staphylococci appears to be the upper respiratory tract and not the gut; however, coagulase-positive strains artificially established in the nose by eating infected food could not be made to invade deep tissue.

LITERATURE CITED

1. Bischoff, F. 1942. Conditions required to produce a prolonged hypothermia in the mouse. *Cancer Res.* 2: 370-371.
2. Boring, W. D., G. M. ZuRhein, and D. L. Walker. 1956. Factors influencing host-virus interactions. II. Alteration of Coxsackie virus infection in adult mice by cold. *Proc. Soc. Exp. Biol. Med.* 93: 273-277.
3. Fröhlich, A. 1938. Des Verhalten des weissen Blutbildes bei allgemeiner Erfrierung. *Deut. Zchr. gericht. Med.* 30: 199-202.
4. Germuth, F. G., Jr. 1956. The role of adrenocortical steroids in infection, immunity and hypersensitivity. *Pharmacol. Rev.* 8: 1-24.
5. Halpern, B. N., P. Dick, G. Biozzi, and G. Mene. 1951. Influence du refroidissement sur l'activité granulopexique du système réticulo-endothélial. *C. rend. Soc. biol.* 145: 503-505.
6. Hardy, J. D. 1950. Physiological responses to heat and cold. *Ann. Rev. Physiol.* 12: 119-144.
7. Hardy, J. D. 1961. Physiology of temperature regulation. *Physiol. Rev.* 41: 521-606.
8. Hemingway, A. 1945. Physiological effects of heat and cold. *Ann. Rev. Physiol.* 7: 163-180.

SECONDARY BACTERIAL INFECTION IN MICE

9. Junge, J. M., and S. M. Rosenthal. 1948. Effect of environmental temperature upon resistance to pneumococcal infection under sulfadiazine therapy and upon body temperature and oxygen consumption during infection. *J. Immunol.* 58: 237-244.
10. Lwoff, A. 1959. Factors influencing the evolution of viral diseases at the cellular level and in the organism. *Bacteriol. Rev.* 23: 109-124.
11. Muschenheim, C., D. R. Duerschner, J. D. Hardy, and A. M. Stoll. 1943. Hypothermia in experimental infections. III. The effect of hypothermia on resistance to experimental pneumococcus infection. *J. Infect. Dis.* 72: 187-196.
12. Previte, J. J., and L. J. Berry. 1962. The effect of environmental temperature on the host-parasite relationship in mice. *J. Infect. Dis.* 110: 201-209.
13. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 27: 493-499.
14. Schaedler, R. W., and R. J. Dubos. 1962. The fecal flora of various strains of mice. Its bearing on their susceptibility to endotoxin. *J. Exp. Med.* 115: 1149-1160.
15. Schmidt, J. R., and A. F. Rasmussen, Jr. 1960. The influence of environmental temperature on the course of experimental herpes simplex infection. *J. Infect. Dis.* 107: 356-360.
16. Schneider, H. A., and N. D. Zinder. 1956. Nutrition of the host and natural resistance to infection. V. An improved assay employing genetic markers in the double strain inoculation test. *J. Exp. Med.* 103: 207-223.
17. Smith, R. E., and D. J. Hoijer. 1962. Metabolism and cellular function in cold acclimation. *Physiol. Rev.* 42: 60-142.
18. Taylor, H. M., and L. Y. Dyrenforth. 1938. Chilling of the body surfaces; its relationship to aural and sinus infections. *JAMA* 111: 1744-1747.

MIRAGLIA AND BERRY

19. Walker, D. L., and W. D. Boring. 1958. Factors influencing host-virus interactions. III. Further studies on the alteration of Cocksackie virus infection in adult mice by environmental temperature. J. Immunol. 80: 39-44.
20. White, C. 1952. The use of ranks in a test for significance for comparing two treatments. Biometrics 8: 33-41.
21. Wilcoxon, F. 1949. Some rapid approximate statistical procedures. American Cyanamid Co., New York.
22. Wildführ, G. 1950. Über die beeinflussung des Alexin-und Opsoningehaltes bei Erkältungsdisponierten durch Kältereize. Arch. Hyg. und Bakteriologie, 133: 49-58.

DISCUSSION

NUNGESTER: Here, again, we have the problem of the hypothermic animal apparently secondarily invaded more frequently than an animal kept at the normal ambient temperature.

MIRAGLIA: Did you say hypothermic animal?

NUNGESTER: Yes.

MIRAGLIA: We try to be very careful to avoid this. The normal controls were not hypothermic, but of course when the animal is infected, the temperature does go down.

BERRY: I would just like to ask an obvious question. Should this be called a secondary infection? If infection implies any disadvantage to the host, I am not sure this is an infection in these mice. It is an interesting observation, however. Dr. Miraglia is attempting to establish coagulase-positive staphylococci in the respiratory tract to see if he can then isolate coagulase-positive staphylococci from the deep tissues. If it can be achieved

SECONDARY BACTERIAL INFECTION IN MICE

with a highly virulent organism, then perhaps a real secondary infection can be established. This is the direction that the work is now taking, so the dream of studying secondary infections may yet come true.

ANDREWES: May I make a rather philosophical comment which applies to all the papers we have heard this morning? It seems to me that in every case, we have been presented with facts which are rather difficult to explain, and I think the reason is that, in these various instances, we don't know exactly why the animal dies when it dies, what kills it, nor do we know what saves the animal when the different mechanisms are operating. It seems to me that in every instance, if only we knew why the animal died and more details about what saved it, we would be able to isolate the various factors and pin down what it is that is affected by chilling. In Dr. Miya's paper, for instance, I was very struck with the fact that from what he reported and from what other people have reported, it didn't look as if chilling had a tremendous effect on the amount of antibody which was formed, and yet in his chilled and unadapted mice, the immunity mechanism, which on paper was perfectly adequate, failed to work. Now, why wouldn't it work; what is the difference between these two groups of mice? If we knew what sort of thing, I think we might understand how cold works. I am not suggesting that we should abandon all work on chilling until we know the answers to these fundamental questions, because I think it is possible that studying the chilling may help us to understand. What we need is for experiments going on in parallel on the mechanism which operates under normal, ordinary circumstances, combined with more experiments on the effect of chilling. I am sorry if all that appears platitudinous.

NUNGESTER: I think it is very appropriate that this statement be made, and this point of view be brought out. Just ask a simple question: why do you have a fever when you have an infection?

CAMPBELL: Well, along the same lines, there are mechanisms of defense other than antibody, of course. So I was wondering what happens to the phagocytic cells and the lymphocytes? There

MIRAGLIA AND BERRY

must have been some studies along this line.

MIRAGLIA: There have been some studies along these lines, and it appears that there is an increase in the number of phagocytes when an animal is subjected to cold, but their capacity to phagocytize and digest is greatly impaired. No, we have tried to mimic the effect of cold by using known RES suppressants, such as Proferrin, and we have been somewhat successful in this, but we need to do a great deal more work.

BERRY: Just by chance, I have a slide. This shows carbon clearance following intravenous injection into mice. Almost complete clearance is accomplished in about thirty minutes. Animals kept at 5° C for two hours and eighteen hours were injected with carbon and compared with mice housed at 25° C. There is not a dramatic difference, but it is statistically significant. We have no other data at the present time which evaluates the effect of cold on the activity of the reticuloendothelial system. I was talking with Derrick Rolly of the University of Adelaide in Montreal, and he suggested that we use labeled bacterial cells rather than carbon; then, he said, we would probably get a completely different type of result. We shall certainly try to do this very promptly now.

NUNGESTER: These results were in what animal, and for how long had it been chilled?

BERRY: The straight line applies to two different groups; mice chilled for two hours, and mice chilled for eighteen hours. The two-hour time period and the eighteen-hour time period gave similar results. We used the two-hour time period because by then the body temperature has dropped.

MIYA: I'd like to answer Sir Christopher. I don't want to sound like I keep harping on this psychological effect, but if you will recall from the slide with respect to the Klebsiella pneumoniae, the isolated mice, singly caged and immunized, were not protected under acute cold stress. These mice are subject to a stress of cold, a stress of isolation, and a stress of challenge, which makes a total of three stresses; whereas, their counter-

SECONDARY BACTERIAL INFECTION IN MICE

parts housed in groups have only the stress of cold, plus the stress of challenge. If you talk in terms of how many stresses are actually applied to the experimental animal, it may be a quantitative thing that you could put your finger on. Let's say it is the function of the total stress as applied to the organism, and I think this can be tested by taking a singly-caged animal and placing it at room temperature in a cage which has an electrical charge to it, then you have the three stresses. You can induce as many more stresses as you see fit.

VIERECK: Dr. Miya, do I understand you to say that isolation is a stress? Usually, in theories of population dynamics, densities, as they get greater, are considered to be more stressful. I know you are dealing with animals in the cold, but it sounded like you considered isolation, per se, without cold, to be a stress to an animal. What is your evidence?

MIYA: Well, I don't have any for an animal, but I think in terms of human beings.

VIERECK: High population density has been studied as a stress in humans.

MIYA: I think I could survive longer in Alaska with one other person to talk to than just by myself.

MITCHELL: Mice are happy only when they're alone.

MIRAGLIA: I don't wish to minimize the psychological effect, but we have conducted experiments with both group-housed and isolated animals at room temperature, and the LD₅₀ is not altered, so using this criteria alone, which, to be sure, is not enough by itself, there is no psychological effect of isolation.

REINHARD: It seems to me that the investigators here are facing the extreme difficulty of separating the effect of one type of flora from another. And you have to go to extreme kinds of manipulations, like feeding hydrochloric acid to rid a beast of one or other organisms. I wonder, in this day and age of germ-free animals, whether the latter would not be more suitable

MIRAGLIA AND BERRY

subjects for the study of the effect of individual species of bacteria in individual animals.

NUNGESTER: That would be very interesting except for the price.

BERRY: The germ-free animal is a highly artificial animal, let me say.

REINHARD: That is true. And so is the animal that is rid of any one part of the flora by the rather rigorous means that have been described.

PREVITE: Getting back to Sir Christopher's comments and Dr. Campbell's comments, in a very light vein I would like to mention that work has been done by some Hungarian workers. They have reported on studies of the complement titers, and phagocytic capacity of Guinea pigs after acclimatization to cold. The animals were housed outdoors during winter and warmer seasons of the year. I was very excited about these papers some time ago. However, their third paper strongly implied that those animals housed outdoors in the cold passed on greater immunity to disease because of the inheritance of acquired characteristics.¹

BERRY: Coming back to Sir Christopher's remark about the immunized mice showing an effect of cold, whereas the non-immunized mice do not. Cold has a subtle influence on host-parasite interaction, and if one is dealing with a highly virulent organism that is certain to kill the animal, then cold will not modify this relationship. If one has a relationship that is more nearly in balance, one that can go either way, then cold can tip the balance and produce an effect either way. The cause of death in an infectious disease is another point, and something that we all need to know more about. There are a few people foolish enough to work on this problem, and I hope more will tackle it, because ultimately, infectious diseases must be understood at a metabolic level. As difficult as it is, I would like

¹ Szemere, Gy., A. Bodi, and L. Csik, 1960. *Academiae Scientiarum Hungaricae Tomus X.*

SECONDARY BACTERIAL INFECTION IN MICE

to make a plea that everyone keep the problem in mind, and whenever there is a chance, throw light on it.

**INFLUENCE OF COLD
ON HOST-PARASITE INTERACTIONS**

PART III

Editor

ELEANOR G. VIERECK

ARCTIC AEROMEDICAL LABORATORY
FORT WAINWRIGHT
ALASKA

1963

COLD AND COLDS

Sir Christopher Andrewes

Overchalke Coombe Bissett
Salisbury, England

ABSTRACT

Two problems arise: (1) Does cold really precipitate colds in individuals? (2) Why are colds commoner in summer? Are these two problems or only one problem? (1) The popular belief that cold or damp can precipitate a cold is very strong. Experiments at the Salisbury Common Cold Unit failed to confirm the notion. Can the belief be due to popular confusion between cause (fall in temperature) and the earliest symptom (a feeling of chilliness); or does this effect of cold only act in certain infrequent individuals or at certain infrequent moments, when virus in a latent state can be activated? Chilling does not seem to activate colds in small isolated communities in the Arctic or elsewhere. (2) Colds are certainly commoner in temperate zones in winter than in summer. Many observers report three waves - one in September-October, one in January, and often a third, lesser one in March. Very many things can be correlated with change in season; but which are the important ones? There is little evidence that temperature is itself the direct cause. Rather better, but still unconvincing, evidence might implicate humidity, perhaps because cold viruses could survive better in the air at low relative humidities. A "winter factor" could operate in such a way, favouring the virus' survival, or indirectly through change in people's habits, again permitting more cross-infections to occur. There may be "conditioned epidemics", a virus such as Influenza being seeded into a population but not manifesting itself until conditions, perhaps meteorological ones, are favourable. Possibly the physiology of the respiratory mucosa is all important. A homeostatic mechanism may not respond promptly enough to environmental changes and virus attack may be favoured at this "unguarded hour". Recent work at Salisbury and elsewhere, permitting identification and titration of "Rhinoviruses" offers hope of a direct attack on some of these questions. Understanding of the natural history of colds may offer better hope of controlling such infections than a programme directed towards specific viruses.

I have not come all the way to Alaska to tell you what the relation between cold and colds is; rather, I have come in hope that in the discussion following my remarks some light may be shed on the obscurity which veils this subject. Why are colds called "colds"? Is it because the subject feels cold, or is it because chilling is thought to precipitate an attack? Or perhaps because colds come at the cold season of the year? We have really to consider two prob-

ANDREWES

lems which may or may not turn out to be one and the same problem: 1) Does chilling really set off a cold infection and if so how? 2) Why are colds commoner in winter than in summer? In other words, is the seasonal prevalence of colds simply due to a summation of the effect of chilling on individuals? At the moment there is good scientific evidence that colds are in fact commoner in winter. Evidence as to the effect on the individual is more doubtful.

Cold and Colds in Individuals

Many people are quite confident that chilling will bring on a cold in them, and they nearly all say that this can happen within an hour or two. A friend of mine has to take time off when she wants her hair washed, as it must be dried immediately and properly, otherwise she "invariably gets a cold". We have offered to test this experimentally but have met with no co-operation.

Experiments were conducted at the Common Cold Research Unit at Salisbury to test the effects of chilling. In this unit, volunteers come for ten days at a time, having offered their services as "human guinea-pigs". They are kept isolated, usually in pairs, and appropriate precautions are taken so that there is no subjective bias in deciding whether or not our experimental procedures have given them a cold. In an experiment several years ago, we took three groups of six volunteers (Andrewes, 1950). The three pairs in one group were given diluted virus, which was expected to produce only a few colds. Another six were soaked in hot baths and then made to stand, undried, in their bathing dresses in a draughty corridor for 30 minutes. After that, they wore wet socks for some hours. A third group of six received the dilute virus plus the chilling treatment. Chilling alone produced no colds. The "Virus alone" group got two colds, while the group with virus plus chilling developed four colds. Interesting, but not statistically significant. We repeated the experiment and again chilling alone did nothing. But in the other two groups the result was reversed; there were fewer colds with chilling than without it. In a third experiment our volunteers went for a walk in the rain; on returning rather cold and tired, they found that we had turned off the heat in their quarters. They were treated in three groups as before, and, as in the second test, chilling was not seen to

COLD AND COLDS

have any effect in predisposing to colds. Dowling et al. (1958) reported similar findings.

There is quite an extensive literature, reviewed by Thomson and Thomson (1932), on the effect on the nasal mucosa of chilling applied locally to the nose, to the whole body, or to the feet. The experiments have mostly been carried out in hope of determining why chilling causes colds. No evidence is adduced on the preliminary question of whether chilling causes colds. Mudd and Grant (1919), amongst others, found that cooling the body surface causes blanching of the upper respiratory mucosa, and a fall in temperature there of rather less than 1° C. Cooling of the feet alone was rather less effective. Different observers are not in agreement as to whether local draughts playing upon the nose are more or less effective than cooling of the body generally. Schmidt and Kairies (1931) confirmed other observers that chilling caused mucosal ischaemia. This happened to everyone they tested, but the rate of return to normal was very variable, a fact having possible bearing on varying susceptibility to colds. There is one report that chilling causes increased acidity in the saliva with a suggestion that nasal secretion may be similarly affected; this, if true, could perhaps be related to the rather acid conditions which common cold viruses seem to like in tissue-culture. Draughts could, of course, operate by causing local desiccation and hence temporary stagnation in the sheet of mucus which is normally flowing continuously backwards over the mucous membranes... But I am in danger of becoming fascinated by these reports of thirty years ago and am being entrapped into discussing "how" and avoiding the question of "whether".

I offer for discussion three possible explanations of the conflict between popular belief in this matter and our own experimental findings.

The first is that the popular belief is a fairy-tale, having no real basis in fact. The second is that people confuse early symptoms with cause. Assuming, as I do, that a common cold is essentially a virus infection, it is hard to explain, on any hypothesis, how this could be "full-blown" within an hour or two of the chilling episode, as is usually reported. Is it not more likely that an early symptom of the virus infection is an undue sensitivity to the effects of the

ANDREWES

physiological adjustments which follow a fall in temperature? Most people not infrequently get wet feet or sit in draughts without feeling chilly and without a subsequent cold; these incidents they forget.

I must digress before putting forward my third explanation. There is evidence as regards several respiratory virus infections, and more particularly influenza, that virus may be widely seeded into a population and yet not give rise to an immediate epidemic. Something has to be right before the outbreak can get started. Particularly remarkable was the way in which the A2, commonly called Asian influenza, spread rapidly in tropical countries, yet was seeded fairly freely into North America and Europe some months before anything very much happened there. With onset of cooler weather, the epidemic broke out. Common cold viruses have occasionally been isolated from normal noses and it is not unlikely that in some people they, like some other viruses, may be in a state of unstable equilibrium with their host, awaiting activation by an appropriate stimulus. We may not have induced in any of our comparatively few volunteers the right kind of equilibrium with the virus we gave them; it may be only a small percentage of the population in which chilling would upset a balance and unleash a cold.

One thing seems fairly certain. In small isolated communities in the Arctic, Antarctic, or elsewhere, cold viruses are lost or else the members of the small community soon become immune to the viruses circulating amongst them (Paul and Freese, 1933). Chilling does not induce colds in them unless there is some contact with the outside world which could possibly introduce fresh viruses. Many of you will be able to tell me if I am misinformed in this matter.

Season and Outbreaks of Colds

Let us now turn to cold and its effects on the incidence of colds in large populations. Every chart I have seen tells the same story: colds are much more frequent in winter than in summer. One observer (Lederer, 1928) asserts that summer colds are more often sporadic and not associated with other cases in the family. In the Northern Hemisphere there is commonly a peak in the incidence of colds with the onset of cooler weather in September and October, a

COLD AND COLDS

second peak early in the New Year, and, less regularly, a third lower peak about March. The New Year peak corresponds to the favourite period for Influenza A epidemics. There are very many differences between summer and winter, not only in temperature and humidity, but in resulting changes in our habits, our dress, and our diet; and it is extremely difficult to pin-point any one of these as responsible for the winter increase in colds. Undoubtedly, a fall in temperature precedes an outbreak of colds, but low temperature as such does not do so; it is the change that matters. Milam and Smillie (1931) found that on the tropical island of St. John, the daily variation over a range of 6.5°C was the same as the difference between the summer and winter maxima (6.5°C to 8°C); yet colds were virtually absent from late May to late October. They thought that with smaller temperature changes, colds were both scarcer and milder than in colder climates. Van Loghem in Holland (1928) and also observers in North America have recorded that outbreaks of colds occur simultaneously over wide areas of the country. It is very difficult to explain this on any theory of simple person-to-person spread. The outbreaks seem rather to be precipitated by temperature changes.

Several writers consider that relative humidity is more important than temperature. Hemmes et al. (1960) reported that the virus of influenza, a winter infection, survived better in the air under conditions of low than of high humidity, whereas the virus of poliomyelitis, a summer disease, behaved in an opposite manner. Hope Simpson (1958) has pointed out that with the onset of colder weather, people light fires indoors, or turn on the central heating, thus causing a considerable drop in relative humidity. At just such times he sees in his practice a sudden increase in upper respiratory infections. He does not venture to suggest whether this could be due to better survival of viruses in the air, as Hemmes' results would indicate, or to some effect on the host's resistance. The effect on virus survival seems unlikely, for rhinoviruses or common cold viruses are closely related in their fundamental properties to the enteroviruses, which include poliovirus; and their stability at various relative humidities resembles that of the poliomyelitis rather than of the influenza virus. So on Hemmes' line of reasoning, colds should be a summer disease. Further, the fall in relative humidity which Hope-Simpson records as happening in the autumn, is only

COLD AND COLDS

second peak early in the New Year, and, less regularly, a third lower peak about March. The New Year peak corresponds to the favourite period for Influenza A epidemics. There are very many differences between summer and winter, not only in temperature and humidity, but in resulting changes in our habits, our dress, and our diet; and it is extremely difficult to pin-point any one of these as responsible for the winter increase in colds. Undoubtedly, a fall in temperature precedes an outbreak of colds, but low temperature as such does not do so; it is the change that matters. Milam and Smillie (1931) found that on the tropical island of St. John, the daily variation over a range of 6.5°C was the same as the difference between the summer and winter maxima (6.5°C to 8°C); yet colds were virtually absent from late May to late October. They thought that with smaller temperature changes, colds were both scarcer and milder than in colder climates. Van Loghem in Holland (1928) and also observers in North America have recorded that outbreaks of colds occur simultaneously over wide areas of the country. It is very difficult to explain this on any theory of simple person-to-person spread. The outbreaks seem rather to be precipitated by temperature changes.

Several writers consider that relative humidity is more important than temperature. Hemmes et al. (1960) reported that the virus of influenza, a winter infection, survived better in the air under conditions of low than of high humidity, whereas the virus of poliomyelitis, a summer disease, behaved in an opposite manner. Hope Simpson (1958) has pointed out that with the onset of colder weather, people light fires indoors, or turn on the central heating, thus causing a considerable drop in relative humidity. At just such times he sees in his practice a sudden increase in upper respiratory infections. He does not venture to suggest whether this could be due to better survival of viruses in the air, as Hemmes' results would indicate, or to some effect on the host's resistance. The effect on virus survival seems unlikely, for rhinoviruses or common cold viruses are closely related in their fundamental properties to the enteroviruses, which include poliovirus; and their stability at various relative humidities resembles that of the poliomyelitis rather than of the influenza virus. So on Hemmes' line of reasoning, colds should be a summer disease. Further, the fall in relative humidity which Hope-Simpson records as happening in the autumn, is only

COLD AND COLDS

second peak early in the New Year, and, less regularly, a third lower peak about March. The New Year peak corresponds to the favourite period for Influenza A epidemics. There are very many differences between summer and winter, not only in temperature and humidity, but in resulting changes in our habits, our dress, and our diet; and it is extremely difficult to pin-point any one of these as responsible for the winter increase in colds. Undoubtedly, a fall in temperature precedes an outbreak of colds, but low temperature as such does not do so; it is the change that matters. Milam and Smillie (1931) found that on the tropical island of St. John, the daily variation over a range of 6.5°C - was the same as the difference between the summer and winter maxima (6.5°C to 8°C); yet colds were virtually absent from late May to late October. They thought that with smaller temperature changes, colds were both scarcer and milder than in colder climates. Van Loghem in Holland (1928) and also observers in North America have recorded that outbreaks of colds occur simultaneously over wide areas of the country. It is very difficult to explain this on any theory of simple person-to-person spread. The outbreaks seem rather to be precipitated by temperature changes.

Several writers consider that relative humidity is more important than temperature. Hemmes et al. (1960) reported that the virus of influenza, a winter infection, survived better in the air under conditions of low than of high humidity, whereas the virus of poliomyelitis, a summer disease, behaved in an opposite manner. Hope Simpson (1958) has pointed out that with the onset of colder weather, people light fires indoors, or turn on the central heating, thus causing a considerable drop in relative humidity. At just such times he sees in his practice a sudden increase in upper respiratory infections. He does not venture to suggest whether this could be due to better survival of viruses in the air, as Hemmes' results would indicate, or to some effect on the host's resistance. The effect on virus survival seems unlikely, for rhinoviruses or common cold viruses are closely related in their fundamental properties to the enteroviruses, which include poliovirus; and their stability at various relative humidities resembles that of the poliomyelitis rather than of the influenza virus. So on Hemmes' line of reasoning, colds should be a summer disease. Further, the fall in relative humidity which Hope-Simpson records as happening in the autumn, is only

ANDREWES

noteworthy in unoccupied rooms. In crowded rooms where cross-infection might be expected to occur, relative humidity is never very low.

What of temperature changes as causing changes in people's habits? Undoubtedly people tend to spend their spare time together indoors in the winter and much more out of doors in summer. This would tend to encourage spread of infection in winter - or so one might at first sight suppose. But if one considers workers in offices, factories, and shops (a considerable part of the population), their lives differ in summer and winter for only a small part of the day. Many of them use crowded public transport all through the year and they are perennially cheek-by-jowl in their shops, factories or offices. Probably there is better ventilation in these surroundings in the summer time, but nothing achieved by students of air hygiene has yet encouraged us to believe that respiratory infections are likely to be greatly reduced by such means; nor yet by U-V irradiation, chemical aerosols or other methods designed to give an equivalent result. The fact is that as regards colds, the experimental evidence available suggests that cross-infection takes place mainly through direct hits with infectious particles at close range rather than through minute droplet nuclei (Lovelock et al., 1952). So ventilation could hardly be expected to play a major role.

There are, of course, some circumstances in which particular kinds of habit changes leading to close aggregations certainly favour spread of virus infections. Quite a number of viruses, some types of adenoviruses, Coxsackie A 21 (or Coe virus), and Influenza B, all cause outbreaks of respiratory infections mainly in recently collected service recruits or in children re-assembling at boarding-schools after holidays. Even here season plays a role, for the adenovirus outbreaks amongst recruits are not important during summer months.

We seem to be frustrated at every turn. Every promising clue seems to peter out. Are recent advances in knowledge about colds and other viruses likely to be able to help us? I think they are. First of all, Tyrrell and his colleagues at Salisbury (Tyrrell et al., 1959) have found out how to cultivate viruses from a high proportion of common colds in adults. These we are calling Rhinoviruses (nose-

COLD AND COLDS

viruses). They can be grown in cultures of human embryonic kidney, and in diploid cell lines from human embryonic lung; a minority of the strains grow also in monkey kidney and other primate cells. The trick is to cultivate at a lower temperature than is conventional (33°C), at a lower pH, and in rolled tubes to give good oxygenation. New developments make their study easier. They grow in a wider range of cells; they can be studied quantitatively by counting the tiny foci of degeneration produced in cell sheets (Parsons and Tyrrell, 1961); even macroscopically visible plaques can now be produced (Porterfield, 1962). These Rhinoviruses resemble enteroviruses in being very small, ether-resistant viruses. They differ in their cultural requirements, greater lability towards acid, habitat, and pathogenicity. They are of many different serological types, and although we don't yet know how many, 30 is probably a low estimate. We are now passing the preliminary stage of establishing that these viruses do, in fact, cause many colds all over the world. The techniques developed can now be applied to studies of epidemiology. Quantitative studies are particularly needed. For many years in our work at Salisbury we could only detect virus by seeing whether or not material under study would produce colds when dropped up the noses of volunteers. Our subjects reacted very variously, and at best we could only infect 50 per cent of them. Quantitative studies were almost impossible. Now that one can count rhinovirus plaques, things are very different. It should not be difficult to discover the whereabouts of virus in the environment, and just when, how, and in what quantity it is shed from an infected person. I should not be wholly surprised to discover that virus shed by a cold-sufferer in summer was quantitatively less than in winter, so that there was less danger of infecting others. All sorts of other quantitative studies should be applicable from now on, including those concerned with seasonal variation in resistance to colds.

Resistance to Colds

Jackson and Dowling (1959) in Chicago produced evidence that resistance to five strains of colds was specific, directed against a particular strain of virus. Discovery of the serological multiplicity of cold viruses fits in with this. Antibodies to particular strains seem to be well correlated with resistance to those strains. Yet other

ANDREWES

evidence suggests that there is a non-specific element in immunity. It is generally agreed that in isolated communities immunity to colds falls, so that contact with civilization after a long interval is quickly followed by colds, and often they are severe ones. Why? The usual explanation is that people in a large community are constantly subjected to little doses of virus which reinforce their immunity, often without giving rise to any symptoms. Yet if there are 150 or 57 or some other large number of cold viruses, it is unlikely that we are all being regularly exposed to all of them. If not, why are we so much more resistant than these lately-isolated people? And why don't they get 57 colds when they begin to mix with society? It seems that if there is a nonspecific as well as a specific immunity, the facts could be reasonably explained. This could be mediated by interferon, a virus-inhibiting protein produced by cells under the stimulus particularly of dead or damaged virus. Its production seems to supply a quickly acting method of halting a virus infection until antibodies can be made and mobilized. Its action is local and nonspecific, being directed not merely against the virus which evoked it. In tissue-culture, rhinoviruses are amongst those most susceptible to its action, and the Salisbury workers are at present engaged on studies in volunteers of a possible role of interferon in cold virus infections.

If I had to offer a working hypothesis as to the effect of cold in favouring colds, it would be along the following lines. The human body, especially the respiratory tract, is exposed to large environmental changes. The complicated anatomy of the nose is part of a homeostatic mechanism designed to protect the lungs from sudden changes. But it may not operate completely and instantaneously. There may be a lag, and during an "unguarded hour", a virus reaching or already in the mucosa, in equilibrium with the host, may snatch its chance. I doubt whether a local fall in temperature alone suffices; it may well be an indirect consequence of temperature fall. Some evidence suggests that interferon production is not so good at lower temperatures, and that is the sort of thing about which I am speaking.

I regard an attack on this difficult matter as the most important phase of our war against respiratory infection. Injecting vaccines against dozens of sero-types of virus seems to be a rather unpromising business. Discovery of the relation between cold and colds

COLD AND COLDS

could lead to measures effective not only against rhinoviruses but against all the other respiratory plagues as well. I feel that the balance may be tipped by something quite small but devilishly elusive. Perhaps our discussions at this symposium will bring forth a clue.

LITERATURE CITED

1. Andrewes, C. H. 1950. Adventures among viruses. III. The puzzle of the common cold. *New England J. Med.* 242: 235.
2. Dowling, H. F., G. G. Jackson, I. G. Spiesman, and T. Inouye. 1958. Transmission of the common cold to volunteers under controlled conditions. IV. The effect of chilling of the subjects upon susceptibility. *Am. J. Hyg.* 68: 59.
3. Hemmes, J. H., K. C. Winkler, and S. M. Kool. 1960. Virus survival as a seasonal factor in influenza and poliomyelitis. *Nature* 188: 430.
4. Jackson, G. G., and H. F. Dowling. 1959. Transmission of the common cold to volunteers under controlled conditions. IV. Specific immunity to the common cold. *J. Clin. Invest.* 38: 762.
5. Lederer, R. 1928. Die "Wintergrippe" der Atmungserkrankungen Wien. *Klin. Wchschr.* 41: 257.
6. Lovelock, J. E., J. S. Porterfield, A. T. Roden, T. Sommerville, and C. H. Andrewes. 1952. Further studies on the natural transmission of the common cold. *Lancet* 2: 657.
7. Milam, D. F., and W. G. Smillie. 1931. A bacteriological study of "colds" on an isolated tropical island (St. John). *J. Exp. Med.* 53: 733.
8. Mudd, S., and S. B. Grant. 1919. Reactions to chilling of the body surface. *J. Med. Res. (Boston)* 40: 53.

ANDREWES

9. Parsons, R., and D. A. J. Tyrrell. 1961. A plaque method for assaying some viruses isolated from common colds. *Nature* 189: 640.
10. Paul, J. H., and H. L. Freese. 1933. An epidemiological and bacteriological study of the common cold in an isolated arctic community (Spitzbergen). *Am. J. Hyg.* 13: 517.
11. Porterfield, J. S. 1962. Titration of some common cold viruses (Rhinoviruses) and their antisera by a plaque method. *Nature* 194: 1044.
12. Schmidt, R., and A. Kairies. 1931. Experimentelle Studien zur Genese den "Erkaltungs-Katarrhe". *Dent. med. Wchnschr.* 2: 1361.
13. Simpson, R. E. Hope. 1958. Common cold: fact and fancy. *Brit. Med. J.* 1: 214.
14. Thomson, D., and R. Thomson. 1932. The common cold. *Ann. Pickett-Thomson Research Lab.* vol. VIII.
15. Tyrrell, D. A. J., M. L. Bynce, G. Hitchcock, H. G. Pereira, C. H. Andrewes, and R. Parsons. 1960. Some virus isolations from common colds. *Lancet* 1: 235.
16. Logham, J. J. van. 1928. An epidemiological contribution to the knowledge of the respiratory diseases. *J. Hyg. (Camb.)* 28: 33.

DISCUSSION

BLAIR: Sir Christopher, I was particularly interested in your viewpoint with regard to the effect of chilling. I never had occasion to do a study of this particular matter with regard to our patients whom we have cooled down to hypothermic levels, and in the last 100 patients I can recall for whom I have been re-

COLD AND COLDS

sponsible, there has not been one single instance of a cold, and furthermore, no pneumonia, so I think that this would support your idea that chilling is probably not of any importance in the development of a cold.

CAMPBELL: Of course, it's been a long time since I have had bacteriology; we used to think that bacteria may be involved here. You didn't mention bacteria at all. I just wondered whether in your studies you had found any correlation between bacterial flora and virus infection?

ANDREWES: Well, I think everybody agrees that in the late stages of the cold, secondary infection with bacteria comes and causes the sinusitis and the yellow muco-purulent stuff. This is generally about in the early stages of the cold; it is very difficult to find anything abnormal about the bacterial flora then, but there is no doubt that in the later stages they do come in and confuse the picture.

CAMPBELL: But no particular type, I mean.

ANDREWES: No, it varies, I think.

WALKER: Have you done any studies on immunity to the common cold? Jackson and Dowling, I believe, report a surprisingly slow development of the immunity.

ANDREWES: Well, yes, we are studying that, and we agree with them that the immunity seems to be specific. We have one of the people working at Salisbury, Dr. Periera, who is the origin of the HGP strain of virus, and he has been bled at intervals. His serum, before he had a certain cold in 1957, had no antibodies to his strain. Then he had this attack of cold and his antibodies came up pretty quickly. Since then, he has been followed along and he has had several colds and we have gotten different viruses from him. The original HGP virus is one of the ones that grew in monkey tissue. One of the other strains was one that didn't grow in monkey, but would grow in human tissues and another was a strain which would transmit colds to volunteers, but we couldn't grow it at all. All this time he has

ANDREWES

had only slightly falling antibody to his original strain and this has been quite unaffected by his attacks of these other colds.

WALKER: And his antibody came up in a matter of a couple of weeks?

ANDREWES: I can't recall, but fairly soon.

WALKER: As I recall, the reports of Jackson and Dowling show that this was much slower in their experience.

ANDREWES: I don't remember their doing it with antibodies.

WALKER: It seems to me they got a peak only after six months or a very prolonged period of time, which is strange.

SCHMIDT: I wonder if you might have a comment on the work that I think has been done by Kruger and others¹ concerning the concentration of the negative or positive charged ions in the atmosphere and its effect on the mucosal lining.

ANDREWES: I am afraid I don't know about that.

TUNEVALL: There is one thing that relates to that season of the year, and that is when our children return from their holiday to school. Couldn't the incidence of colds perhaps be correlated to these instances when the children get together?

ANDREWES: Well, there is no doubt that children are very much more effective spreaders of infection than adults. We carried out some epidemiological studies in a rural valley near Salisbury, and it was found that colds in adults in families in which there were school-age children were two and a half times as common as in families when there were no school-age children. There is no doubt that the little darlings spread the virus very efficiently.

¹ Krueger et al. 1959. Proc. Soc. Exp. Biol. Med. 102: 355-357.

COLD AND COLDS

SULKIN: You mentioned that to limit the occurrence of the common cold through classical immunization might present a difficult problem because of the growing number of serologic types. I wonder, in your opinion, if it is conceivable that a new type of immunization procedure might be evolved through the mechanism of interference. That is to say, since inactive viral particles will produce interferon, is it conceivable that by introduction of say, influenza virus into the external nares, that one might manufacture sufficient interferon to cope with any one of these common cold viruses?

ANDREWES: We have actually tried that and it didn't come off, but we don't despair of an approach on that line. Issacs found that the production of interferon is not only mediated by virus approach; he thinks it is fundamentally a reaction to any foreign nucleo-protein approach, and it is possible to produce some interferon with nucleo-protein approaches of non-viral origin. One man made an interesting suggestion which we haven't followed up; that is, that the low incidence of colds in the summer might be due to the fact that in the summer, people are constantly being stimulated with nucleo-protein produced from pollen.

NUNGESTER: I hate to agree with Dr. Blair and sell Joe Berry's long underwear short here, and I wonder if you believe that experiments with humans where there is more or less uniform cooling are characteristically comparable to the sort of things that you have done at Salisbury. In keeping with the work of Mudgrant and Goldman,² it isn't the uniform cooling of the whole body that is important, but the irregular cooling of parts of the body that is important.

WALKER: And I would add, to the right temperature.

NUNGESTER: Yes.

ANDREWES: Our feeling about this effect of cold is that the

² Annals of Otology, Rhinology, and Laryngology. 30:1. 1921.

COLD AND COLDS

SULKIN: You mentioned that to limit the occurrence of the common cold through classical immunization might present a difficult problem because of the growing number of serologic types. I wonder, in your opinion, if it is conceivable that a new type of immunization procedure might be evolved through the mechanism of interference. That is to say, since inactive viral particles will produce interferon, is it conceivable that by introduction of say, influenza virus into the external nares, that one might manufacture sufficient interferon to cope with any one of these common cold viruses?

ANDREWES: We have actually tried that and it didn't come off, but we don't despair of an approach on that line. Issacs found that the production of interferon is not only mediated by virus approach; he thinks it is fundamentally a reaction to any foreign nucleo-protein approach, and it is possible to produce some interferon with nucleo-protein approaches of non-viral origin. One man made an interesting suggestion which we haven't followed up; that is, that the low incidence of colds in the summer might be due to the fact that in the summer, people are constantly being stimulated with nucleo-protein produced from pollen.

NUNGESTER: I hate to agree with Dr. Blair and sell Joe Berry's long underwear short here, and I wonder if you believe that experiments with humans where there is more or less uniform cooling are characteristically comparable to the sort of things that you have done at Salisbury. In keeping with the work of Mudgrant and Goldman,² it isn't the uniform cooling of the whole body that is important, but the irregular cooling of parts of the body that is important.

WALKER: And I would add, to the right temperature.

NUNGESTER: Yes.

ANDREWES: Our feeling about this effect of cold is that the

² Annals of Otology, Rhinology, and Laryngology. 30: 1. 1921.

ANDREWES

way we did the experiment it didn't show anything, but we are not willing to say that there isn't a relation. We failed to achieve it, and we hope other people will find a way they can do it; then we will believe them. We are not biased about the whole thing.

NUNGESTER: A few years ago, we got to playing with this sort of thing. In the process of taking nasal washings, we took just a little bit of a history of the people from whom we took the nasal washings, if they had a cold. These are students, of course; they like to brag on how hard they have been working, so you have to discount this a bit, but it seems that there was almost a pretty good correlation between loss of sleep and the incidence of a common cold. There seems to be more of a correlation with this sort of fatigue and stress than there was with exposure to cold. Have you had any experience with fatigue? Of course, you have told us that you brought the fatigue element in.

ANDREWES: Yes, also the opposite effect; if people go away for a summer holiday and come back feeling absolutely on top of the world, they're likely to get a cold almost at once.

SCHMIDT: In connection with humidity and its possible effect, it occurred to us, during our studies here in Alaska, that it might be involved. Humidity is very low during the winter months in Central Alaska, to the extent that you waken with a very dry and crusty throat. It is hard to imagine that one wouldn't be most susceptible to any sort of respiratory illness under these conditions. When Dr. Beard, of the Armed Forces Epidemiological Board (at that time, at least) was here, I discussed this aspect with him and he indicated that he had considered this to be a factor some years ago. He investigated this by working in a desert area where it was warm but very dry, and he didn't observe a higher incidence of infection in his subjects. Although our attempts were rather clumsy and humble, no relationship could be established between the relative humidity and upper respiratory infection. Even though you are extremely uncomfortable, you seem to survive very nicely.

ANDREWES: Of course, under those desert conditions is the

COLD AND COLDS

time when you get epidemics of cerebral spinal fever. I think that seems to go in the Sudan and other regions around the Sahara.

CAMPBELL: I would like to ask a fundamental question. It has nothing to do with cold exposure, but in immunization to viruses that seem like polio, you have to have the specific strain to produce immunity. We are so conditioned with the pneumococcus work in which the polysaccharide plays a major role in infection. How do you envision the structure of a virus that is so specific? There must be something in common with all these strains of cold viruses, and if we had enough, you probably could show immunologically or serologically that there were cross reactions, like the C substance in pneumococcus. Do you envision a capsule or something around the virus?

ANDREWES: Most of these viruses consist of nucleo-protein core and the other protein outside, and in the case of the smaller viruses, that is about all there is to it. We have found evidence of a very slight amount of cross-reaction between some of these colds. It is only very trivial, but there are small amounts, as in the case of the pox group of viruses covering not only all the animal poxes, but also myxoma and a number of others. It has quite recently been shown by Japanese workers, and by Fenner in Australia, that there is a common nucleo-protein antigen which was overlooked for many years. I wouldn't at all think it impossible that a similar thing would be found with some of these smaller viruses. Whether it would be of any use in inducing immunity would be anybody's guess.

WALKER: Most commonly, this nucleo-protein represents the common antigen of the group. It is not a protective antibody.

CAMPBELL: Well, you could modify it some way. They must have something in common. They like to live in the nose; diphtheria likes to live a little further down, but both bacteria and viruses, of course, are local; that's where you get the term neurotropic and dermatropic.

ANDREWES: We need to collaborate with an immunochemist.

ANDREWES

CAMPBELL: Next year, I will be over.

NORTHEY: I'd like to go one step further. How do you feel that antibody really acts in these infections?

ANDREWES: Well, it seems to play more of a part than we expected. With some of these other respiratory infections, you seem to be able to get repeated infections in spite of the presence of some antibody in the serum, but in the case of the common cold viruses, it appears that if you have got an antibody of that particular strain, you are likely to resist challenge by that particular strain. We were really surprised to find that there did seem to be considerable relation between antibody and immunity.

METCALF: Is there a difference between the antibody titer that you find in the serum and the nasal secretions, and if so, is this likely to be of any significance?

ANDREWES: Well, we have looked for neutralizing things in the nasal secretion, but that was some time ago before we really got on to the way to grow these viruses, so I don't think we know the answer to that question. In the case of influenza, of course, you do get the same kind of antibody in the nasal secretions as you do in the serum, but much less of it.

METCALF: I was thinking of the obvious relationship of a fluid bathing a vulnerable point of cells subject to attack.

ANDREWES: Of course, all the earlier work in that is muddled up by the fact that people didn't appreciate the possible presence of interferon.

PREVITE: Just one naive question, not being very familiar with virology. We often hear that there are many different viruses capable of causing what we call the "common" cold. Has anyone made any effort to ascertain how great and variable are the number of viruses in any given area?

ANDREWES: There have been a number of studies of that. Dr. Hamre in Chicago has published some work on that, and it

COLD AND COLDS

appears that it depends on what age group you take. If you take small children, you will find that the rare influenza viruses produce quite a lot of infection. In any age group, the respiratory syncytial virus may be prevalent in one year, and in another year it may be completely absent. Rhinoviruses seem to be the hard core which cause more colds than anything else, but I should make it clear that there are still a great many colds from which we haven't been able to cultivate any viruses, although these things will still produce colds in volunteers. So there is still quite a lot to learn, but I think the proportion of the colds caused by different agents probably varies very much from time to time and from place to place.

Well, I don't know that anybody has actually solved all my more difficult problems for me, but anyway, I am very grateful for all the suggestions that have been made in the matter.

EFFECT OF ENVIRONMENTAL TEMPERATURE ON VIRAL INFECTION¹

Duard L. Walker, M. D.

University of Wisconsin
Medical School
Madison 6, Wisconsin

ABSTRACT

In considering the effects of cold on viral infections, four questions seem to be of particular importance. These are: (1) Can exposure to cold cause an acute but mild and inapparent infection to become an apparent and severe disease? (2) Can it seriously worsen an apparent viral infection? (3) Can it activate a latent viral infection? (4) What are the mechanisms by which cold exerts an effect on viral infections? Indication that the answers to the first three questions can be "yes" is available from studies of infections in animals. Studies in this laboratory on Coxsackie infections in mice are pertinent to the first question. In infant mice the Conn.-5 strain of Coxsackie B-1 virus causes a generalized, lethal infection, but in adult mice the infection is limited to a mild, inapparent pancreatitis. Exposure of adult mice to a 4° C environment, however, results in illness with essentially 100 per cent mortality. Pertinent to the second question are studies on the myxoma-fibroma viruses. Marshall has shown that exposure to cold increases the severity of disease in rabbits infected with attenuated strains of myxoma virus. Relative to the third question, Shope has found that exposure to cold weather appears to activate latent infections of swine influenza virus in swine. Although the mechanisms by which cold exerts an effect on viral infections have not been studied extensively, there is growing evidence that it may be by simply lowering tissue temperature to one more favorable for multiplication of the infecting virus. At normal temperatures Conn.-5 Coxsackie virus multiplies only in the pancreas of the adult mouse. Exposure to 4° C results in reduction of body temperature by 1.0° C to 1.5° C and in multiplication of virus in many organs. Exposure of mice to 36° C raises their body temperature 2° C and inhibits multiplication in all tissues, including the pancreas. Study of Conn.-5 virus multiplication in *in vitro* cultures reveals that the virus does not multiply well in adult mouse tissues at 37° C or at higher temperatures, but does multiply well at 35° C.

¹ Studies by the author on this subject were supported by grants from the National Institute of Allergy and Infectious Diseases and the National Cancer Institute.

WALKER

Studies concerned with the effect of environmental temperature on viral infections have sometimes been confusing and seemingly contradictory, but cold has most often been found to aggravate viral infections. For the purposes of this symposium, then, it seems to me that there are four questions that should be considered: 1) Can exposure to cold cause a mild, inapparent viral disease? 2) Can this exposure seriously worsen an apparent viral infection? 3) Can it activate a latent viral infection? 4) What are the mechanisms by which cold exerts its effect on viral infections?

It is difficult to find direct experimental data bearing on these questions that have been obtained in studies of infections in man, but some evidence is available from studies in animals, and some information is available from study of viral infection of cells in culture. The data are not extensive, but I think they provide indication that the answers to the first three questions can be yes, at least with selected virus-host systems and under laboratory conditions, perhaps even in man, and that we may find such effects if we make the proper search.

I should like, then, to disclose this evidence. Some of it is from others, but I shall limit consideration to that which I think has a quite direct bearing on the questions that I have posed.

EXAMPLES OF THE EFFECT OF COLD ON VIRAL INFECTION

Can Exposure to Cold Cause a Mild, Inapparent Viral Infection to Become an Apparent and Serious Disease?

Boring and I have studied a model viral infection in mice caused by the Conn.-5 strain of type B1 Coxsackie virus (Boring, ZuRhein, and Walker, 1956; Walker and Boring, 1958). Although this virus produces a generalized and lethal infection in infant mice, it causes in adult Swiss mice only a pancreatitis. The mice seldom show outward signs of illness even after very large inocula. However, when

TEMPERATURE AND VIRAL INFECTION

Inoculum (i. p.)	Environmental temperature	Deaths**/no. inoc.
Virus Suspension*	25° C	0/20
"	4° C	19/20
Normal mouse tissue suspension	25° C	0/20
"	4° C	0/20

Figure 1. Lethal effect of Coxsackie virus infection in adult mice at 4° C. *4000 LD₅₀ for infant mice. **No. of mice dying during 10 days of observation.

Ambient temperature C		Deaths/no. inoculated
Before inoculation*	After inoculation	
4° for 2 days	25°	0/12**
25°	4°, 1 day, then 25°	0/12
25°	4°, 2 days, then 25°	0/12
25°	4°	12/12
25°	25°, 1 day, then 4°	12/12
25°	25°, 2 days, then 4°	9/12
25°	25°, 4 days, then 4°	9/12
25°	25°, 6 days, then 4°	0/12

Figure 2. Relationship of time of exposure to cold and lethal effect of Coxsackie virus in adult mice. *1500 infant LD₅₀ given i. p. **Deaths in 10 day observation period after mice placed at 4° C.

WALKER

inoculated mice are placed in a room at a temperature of 4° C, the infection then becomes quite uniformly lethal. Figure 1 shows a typical experiment. Acute, limited exposure to cold is not sufficient to change this infection in adult mice from a restricted, asymptomatic one into a fatal infection. Continued exposure through several days is necessary (Fig. 2).

That the deaths of inoculated mice at 4° C are related to viral infection can be shown by neutralizing the virus with specific anti-serum prior to injection or by passively immunizing the mice prior to inoculation. This prevents the deaths at 4° C. In addition, it can be shown that this phenomenon is not caused simply by inability of mice with pancreatitis to survive in the cold, but is due to a real enhancement of the infection. Measurement of virus levels and study of tissue histology indicate that at ordinary temperatures viral multiplication is limited to the pancreas, but that in mice at 4° C, viral multiplication and tissue damage takes place in many tissues. Data on this point will be presented in a later section.

An investigation of Briody and associates (Briody et al., 1953) concerned with what could well be called inapparent infection is also pertinent here. These workers examined the effect of cold on the process of adaptation of influenza A' virus to multiplication in the lungs of mice. Unadapted influenza virus usually multiplies to some extent in mouse lungs, but it causes little pneumonia or mortality until after a series of serial passages has resulted in a selection of virus capable of rapid and abundant multiplication in the lungs of mice. When inoculated mice were maintained at 5° C, however, the virus grew to 100-fold higher levels, the extent of pneumonia was increased and mice began to die of influenzal pneumonia after only a few passages.

Can Exposure to Cold Seriously Worsen an Apparent Viral Infection?

This question, of course, is not very different from the previous one, because in many instances the difference between inapparent and apparent infection is only one of degree. Nevertheless, the question serves well to introduce work on myxoma virus infections.

TEMPERATURE AND VIRAL INFECTION

The effect of environmental temperature on viral infection has appeared to be of some practical importance in Australia in the evolution of myxomatosis in wild rabbits. Marshall (1959) noted that there was repeated suggestion that myxomatosis spreading naturally through wild rabbits was more lethal in winter than in summer, and Mykytowycz (1956) observed that rabbits experimentally infected with an attenuated strain and housed in unheated quarters had a higher mortality rate in winter than in summer. To test the possibility that these observations were related to ambient temperature, Marshall exposed inoculated rabbits to fluctuating cold (-1°C to $+1^{\circ}\text{C}$ for 16 hours and 15°C for 8 hours each day) and compared the results with those at normal room temperatures (20°C to 22°C) and at elevated temperatures (37°C to 39°C for 16 hours and 26°C for 8 hours each day). These fluctuating temperatures were chosen to simulate day and night fluctuations of winter and summer. He found that ambient temperature had little effect on infections with a highly virulent strain of myxoma virus or with the quite virulent rabbit pox. But if rabbits were inoculated with an attenuated strain of myxoma virus that at 22°C caused death of about 60 per cent of rabbits, then exposure to cold increased the mortality to over 90 per cent and exposure to heat reduced mortality to about 30 per cent (Fig. 3). Myxomatosis in a rabbit is a very distinctive disease, and comparison of the signs of disease in the rabbits at the various temperatures, as well as differences in the levels of virus in the blood, were fairly convincing indications that the differences in mortality rate were due to alterations of the extent and severity of infection instead of to some other effect of the temperatures.

Somewhat similarly, Sulkin (1945) has shown that after inoculation with influenza A virus, mice maintained at 15.5°C have significantly more pulmonary consolidation and mice at 35°C less consolidation than do mice held at 21°C to 25°C . And I judge from the abstracts of Drs. Metcalf and Marcus that they will be providing additional data pertinent to this question.

Can Exposure to Cold Activate a Latent Infection?

Latent here means an inapparent infection that exhibits chronicity and some degree of host-virus equilibrium. Although this is a par-

	Ambient temperature		
	-1° C to +1° C for 16 hrs; 15° C for 8 hrs per day	20° C-22° C	37° C to 39° C for 16 hrs; 26° C for 8 hrs per day
Infected rabbits	36/39 (92%)	22/35 (63%)	9/30 (30%)
Uninoculated control rabbits	0/36		0/31

Figure 3. Mortality in rabbits infected with an attenuated strain of myxoma virus and held at different temperatures. (M irsh.11, 1959).

TEMPERATURE AND VIRAL INFECTION

ticularly interesting question, there seems to be relatively little data that concerns such infections and involves hosts and viruses that lend themselves to detailed study. Dr. Andrewes has already dealt with the common cold in man. There is one study, however, that should be mentioned here.

Shope has pointed out that circumstantial evidence concerned with epizootics of swine influenza has long indicated that the stimulus responsible for precipitating attacks of the disease in swine latently infected with the virus is in some way associated with sudden changes in weather and especially with the onset of cold, wet weather. He performed an experiment (Shope, 1955) in which he prepared 25 swine by feeding them earthworms containing lungworm larvae carrying swine influenza virus. After about 30 days, during which the animals remained well, he then exposed them for from 4 to 24 hours to adverse weather conditions, which always included rain or snow and low temperatures. No data were obtained on the effect of this on the body temperatures of the animals. Eight uninoculated controls and 15 inoculated animals remained well, but 4 swine did develop the typical illness of swine influenza and 6 others developed serological evidence of infection.

POSSIBLE MECHANISMS FOR THE EFFECT OF COLD ON VIRAL INFECTIONS

The studies that I have outlined indicate that with some hosts and viruses under proper circumstances cold can aggravate and activate viral infections. I have limited discussion here to those studies that do point to these possibilities, because I think failures to find an aggravating effect of cold, or even the occasional report of the opposite effect, are only to be expected with some host-virus combinations and under certain circumstances. But the fact that there are a good many reports of no effect or a protective effect of cold must be kept in mind in any evaluation of possible mechanisms. I shall discuss this further in a later section.

WALKER

In any consideration of mechanisms that might account for the effects of cold on viral infection two possibilities quickly come to mind. One is that host defenses are modified by exposure to cold, and the second is that cold acts as a stressing agent and modifies the host through alteration of hormone balance.

The host defense most frequently considered and seems particularly pertinent in viral infection is antibody production. The effect of cold on antibody production has been discussed by others in this meeting, but I want to point out that Marshall (1959) considered the possibility of inhibition of antibody production in his study of rabbit myxomatosis and showed that under the conditions of his experiments antibody against sheep erythrocytes developed as rapidly and to as high levels in rabbits exposed to cold as in control rabbits.

In our study of Cocksackie virus infections in mice, Boring and I have never actually measured the antibody developed against Cocksackie virus in mice at 4°C, because we found that cold still exerted its effect even if we delayed exposure of infected mice until the antibody producing process was well under way (Boring et al., 1956). Specific neutralizing antibody appears in the blood of mice on the third day after inoculation with small quantities of Cocksackie B1 virus and on the fourth day the antibody is at substantial levels (Fig. 4; Boring and Walker, unpublished data). Even though exposure to cold is delayed until the fourth day, its effect is not nullified (Fig. 2). This suggests to us that even if cold were found to have an inhibiting effect on antibody production, this would still not explain the influence of cold on the infection. In addition to this, current work using mice thymectomized at birth indicates that an adult mouse does not develop a generalized lethal infection with Cocksackie B1 virus even though the mouse is incapable of producing antibody.

Other host defenses such as non-specific viral inhibitors and interferon may conceivably be altered by cold, but there is little positive evidence for this, as yet.

The role of stress is difficult to assess. There is little question that the cold exposure used in the studies that I have discussed were of a degree sufficient to cause some of the physiological changes identified with stress. And treatment of animals with large doses of

TEMPERATURE AND VIRAL INFECTION

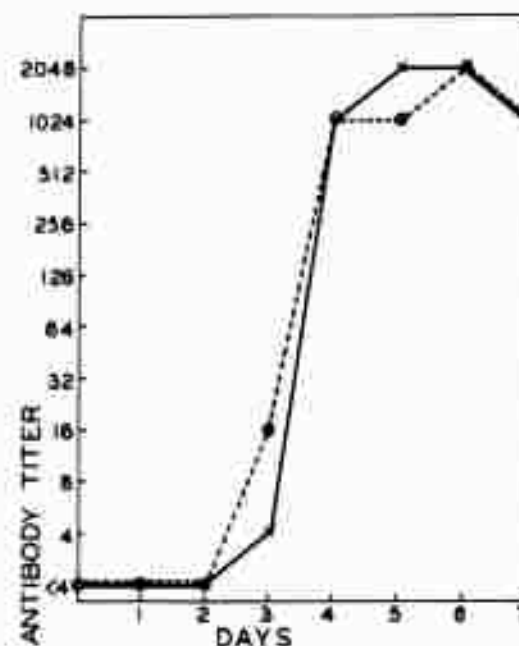


Figure 4. Antibody response to Coxsackie virus infection in mice at 25° C ambient temperature. Adult Swiss mice were given 280 infant mouse LD₅₀ i. p. The antibody titers from two experiments are plotted as separate curves. Antibody assays were made in infant mice and antibody titers are expressed as the reciprocal of the serum dilution that neutralized LD₅₀ of virus.

cortisone causes an aggravation of a number of viral infections similar to that seen with exposure to cold. Administration of 2.5 mg of cortisone to adult mice infected with Coxsackie B1 virus results in a generalized and lethal infection quite similar to that produced by exposure to cold (Boring, Angevine, and Walker, 1955). But Boring and I (1958) were not able to produce similar effects by treating mice with physiologically active doses of ACTH. In addition to this, the fact that short exposures to cold were not effective, that adaptation of mice to cold did not nullify the effect, and that exposure to the stress of heat caused an entirely different response has led us to think that just the stressing effect of cold cannot account for its effect on viral infection.

I have indicated some skepticism that inhibition of antibody pro-

WALKER

Tissue	Day of infection	Virus titers		
		4° C	25° C	36° C
Blood	2	10 ^{-4.3}	10 ^{-4.6}	< 10 ^{-1.0}
	4	10 ^{-3.7}	< 10 ^{-1.0}	< 10 ^{-1.0}
Brain	2	10 ^{-1.5}	10 ^{-1.5}	< 10 ^{-1.0}
	4	10 ^{-2.1}	< 10 ^{-1.0}	< 10 ^{-1.0}
Pancreas	2	10 ^{-6.9}	10 ^{-7.7}	< 10 ^{-1.0}
	4	10 ^{-6.7}	10 ^{-5.7}	< 10 ^{-1.0}
Heart	2	10 ^{-3.75}	10 ^{-3.5}	< 10 ^{-1.0}
	4	10 ^{-4.4}	< 10 ^{-1.0}	< 10 ^{-1.0}
Liver	2	10 ^{-4.5}	10 ^{-4.0}	< 10 ^{-1.0}
	4	10 ^{-5.0}	< 10 ^{-1.0}	< 10 ^{-1.0}

Figure 5. Virus in tissues of adult mice infected with Coxsackie virus* and held at 4° C, 25° C, or 36° C. *Inoculated with 140 infant mouse LD₅₀ i. p.

duction or stress can account for the effect of cold on viral infections. For discussion of another possible mechanism I want to return to the model of Coxsackie virus infections in mice. In studying the effect of cold on this infection, Boring and I (1958) followed the fate of virus in various tissues of infected mice. It was evident that in mice at normal temperatures an initial viremia was followed by significant viral multiplication only in the pancreas. But in mice at 4° C, the virus multiplied to relatively high levels and produced marked damage in several tissues. It was also found that if mice were maintained at an elevated ambient temperature, viral multiplication was inhibited in all tissues, including the pancreas (Fig. 5). In fact, even if the pancreatitis was allowed to progress for 36 hours after inoculation, exposure to 36° C still brought about a prompt drop in virus titer in the pancreas and rapid elimination of the virus. An important point is that when rectal temperatures of mice are measured during exposure to such temperatures, it can be

TEMPERATURE AND VIRAL INFECTION

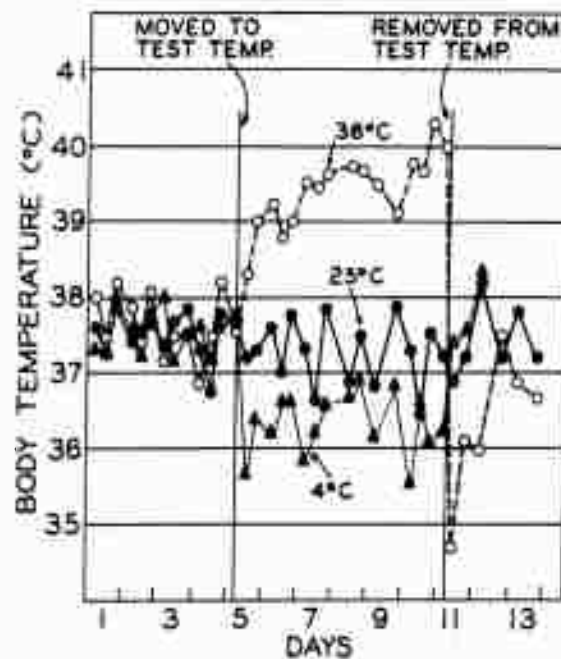


Figure 6. Effect of environmental temperature on the body temperature of mice. Each point represents the mean of the rectal temperatures of 10 mice. (Walker, D. L., and Boring, W. D. 1958. *J. Immunol.* 80: 39-44.)

demonstrated that at 4° C the mouse's internal temperature is lowered about 1° C to 2° C and exposure to 36° C raises the rectal temperature about 2° C to 3° C (Fig. 6).

These phenomena suggest that in the host cell there are temperature-sensitive reactions that can control Coxsackie virus multiplication. Additional support for this has been provided by Boring and Levy (1962) who demonstrated that in HeLa cells *in vitro* the optimum temperature for multiplication of the Conn.-5 strain of Coxsackie virus is 36° C and that multiplication is markedly inhibited at 38° C to 39° C, and is also reduced at temperatures below

WALKER

Temperature of incubation	Virus	
	Fibroma	Myxoma
29° C	5.5*	5.5
33° C	7.5	6.5
35° C	7.5	7.5
37° C	6.5	7.5
40° C	4.5	7.5

Figure 7. Multiplication of myxoma and fibroma viruses in primary rabbit kidney cells at various temperatures. *Log rabbit skin infectious units per ml produced in 48 hours.

36° C. We have recently been looking at this more directly by comparing the multiplication of B1 Coxsackie virus in primary cultures of adult and infant mouse tissues at various temperatures in order to determine the optimum temperature for multiplication in the tissues usually affected in the mouse. Our preliminary results suggest that the virus can multiply in adult tissues if the temperature of incubation is reduced to 35° C and that it is inhibited at higher temperatures, whereas in infant tissues it multiplies to high titer at 37° C to 38° C as well as at lower temperatures. Our experiments have not proceeded far enough, however, to provide really reliable data.

I have already indicated that in his study of myxoma virus infections in rabbits, Marshall (1959) found a pattern similar to the one I have described for Coxsackie virus; that is, an ameliorating effect of high temperature as well as enhancement of the infection by cold. Similar protective effects of elevated ambient temperature had previously been reported by Thompson (1938). Both Thompson and Marshall found that rabbits at test temperatures had shifts of their rectal temperatures of only about 0.5° C downward in the cold and 0.5° C to 1° C upward in the hot room, but their skin temperatures changed 4° C to 5° C. The possibility that these temperature changes may have been important in controlling the course of the infection is supported by other evidence indicating that multiplication of myxoma and fibroma viruses is easily affected by change of temperature. The myxoma and fibroma viruses and their various vari-

TEMPERATURE AND VIRAL INFECTION

ants present a spectrum of viruses that are very closely related in many physical and biological characteristics, including antigenic makeup. However, after intradermal inoculation virulent myxoma virus invades, causes generalized disease, and is almost 100 per cent lethal for domestic rabbits, while fibroma virus causes only local benign tumors that eventually regress without any apparent harm to the rabbit. It is noteworthy that the only tissues in which fibroma will multiply and cause lesions in the adult rabbit, even if injected intraperitoneally or intravenously, are the surface tissues of skin and testes. It can be demonstrated quite easily that myxoma and fibroma viruses differ markedly in their capacity to multiply at temperatures above 35° C. Thompson (1938) demonstrated this in vivo when he raised the skin temperature of rabbits by exposure to heat and showed that fibroma lesions were quite easily inhibited while the disease caused by virulent myxoma virus required higher temperatures to bring about amelioration. This can be shown in primary cultures of rabbit tissues in vitro. Kilham (1959) has provided some data on this, and some of our own data are shown in Figure 7. Fully virulent myxoma virus multiplies to high titer even at temperatures of 40° C to 41° C, while fibroma virus reaches peak titers at 32° C to 35° C and is inhibited to some extent at temperatures as low as 36° C to 37° C and is severely inhibited at higher temperatures. Variants of myxoma virus exist that are reduced in their virulence and are intermediate between myxoma and fibroma viruses in the characteristics of the disease that they produce in rabbits. It was one of these that Marshall used in his study. We are currently comparing the capacity of myxoma virus strains to multiply at various temperatures and their relative invasiveness and virulence in rabbits. This work has not progressed far, but it appears that for many attenuated strains reduction in virulence is accompanied by decreased capacity to multiply at temperatures comparable to the internal temperature of the rabbit.

Other studies indicating a relationship between virulence and capacity to multiply at temperatures above 37° C can be cited. Bedson and Dumbell (1961) have shown this relationship with several poxviruses and their virulence for chicken embryos. Most detailed, however, have been the extensive studies of Lwoff and associates on poliovirus (Lwoff, 1959; Lwoff and Lwoff, 1960, 1961). Lwoff has demonstrated in cells in culture that poliovirus is able to multiply

WALKER

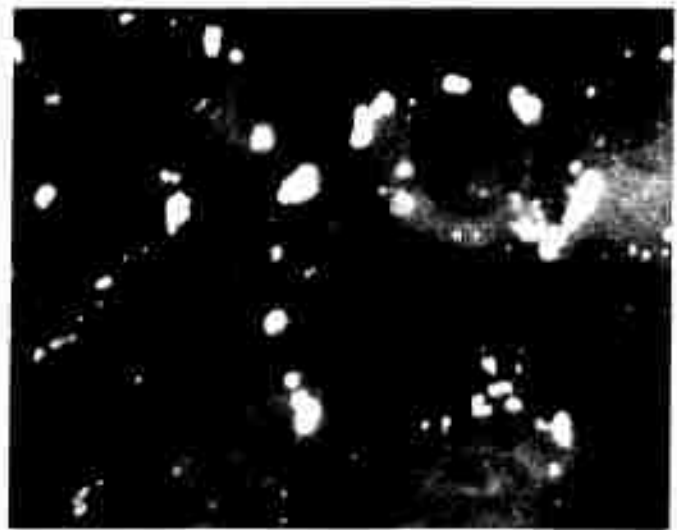
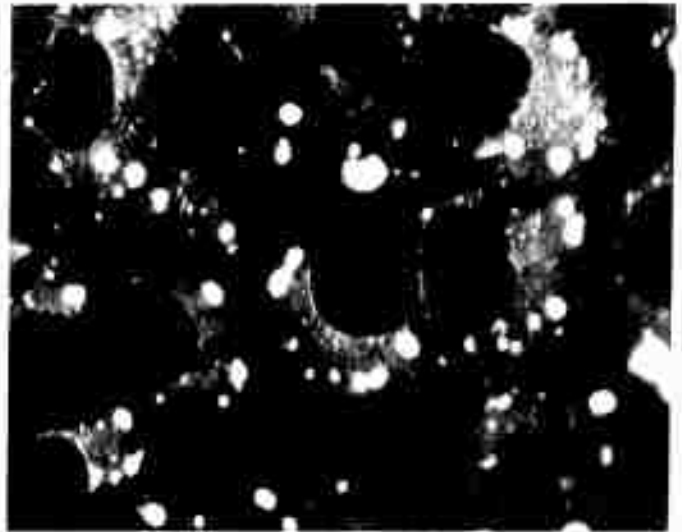
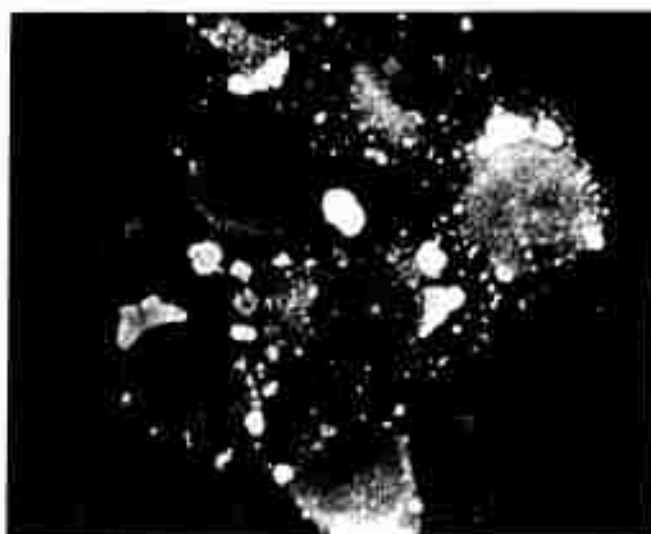


Figure 8. Carrier cultures of mumps virus in human conjunctiva cells (C-M cultures) at different temperatures. Cultures were grown at 37°C and then moved to test tempera-

TEMPERATURE AND VIRAL INFECTION



tures for 24 hours. Cells fixed and stained with fluorescein-conjugated anti-mumps serum. A, 37° C. B, 40° C. C, 35° C. D, 33° C. Magnification -X 1000.

WALKER

only within certain limits of temperature. For many strains a temperature of 40° C or above inhibits multiplication and viral production is depressed below 33° C. It appears that elevated temperature produces a block in the second half of the virus multiplication cycle and low temperatures block the first part of the cycle. But he points out (also Dubes and Wenner, 1957) that strains vary in the temperature that is optimum for their multiplication and that, in general, neurovirulence in monkeys is associated with those strains that are capable of good multiplication at 40° C, and lack of virulence with those incapable of multiplication at 37° C or above.

There is additional evidence from quite a number of studies using tissue cultures or chicken embryos (Enders and Pearson, 1941; Thompson and Coates, 1942; Sharpe, 1958; Hoggan and Roizman, 1959; Wheeler and Canby, 1959) that the temperature range within which viruses can multiply vigorously is often quite limited, and that the upper limit in particular may be quite sharp and abrupt. For several of the viruses studied the optimum temperature for multiplication is a degree or two below that of the internal body temperature of some of the common mammalian hosts. I want to mention briefly some data concerned with mumps virus, because it could conceivably provide some insight into how cold could affect latent infections.

Hinze and I have been studying a carrier system of mumps virus in human conjunctiva cells. This system has been maintained for several years (Walker and Hinze, in press). The cells multiply at a rate comparable to control cultures, and show little evidence of deleterious effect even though use of fluorescent antibody demonstrates that 90 per cent or more of the cells contain viral antigen. As routine, these cultures are grown at 37° C, at which temperature about 1 of every 100 cells appears to be excreting virus as judged by the fact that erythrocytes will adsorb to the cell surface. There is a low level of virus in the medium. At 37° C practically all of the cells contain antigen, but it is restricted to a few sharply outlined, discrete, masses in the cytoplasm (Fig. 8a). At 40° C the antigen is perhaps even more restricted in its distribution (Fig. 8b). But at 35° C or 33° C the antigen becomes widely distributed in the cytoplasm in small granules (Fig. 8c and 8d) and the cells tend to become rounded and ragged in appearance. At 35° C or 33° C erythro-

TEMPERATURE AND VIRAL INFECTION

cytes will adsorb to 50 per cent or more of cells and the virus concentration in the medium increases by 10 to 100-fold indicating that at the lower temperatures the equilibrium between cells and virus is upset and there is much more virus production and release in the cultures.

Lwoff (1959) has emphasized the narrow zone of optimum temperature for viral multiplication and the inhibitory effect of elevated temperature to argue that fever may be an important host defense mechanism in choking off viral infection, and he has pointed to the variation in optimum temperature among virus strains as a partial explanation of variation in virulence. It seems to me that these phenomena can also explain some of the observed effects of cold on viral infection and, indeed, may even account for the variability of observations. I suggest that one can expect to see an effect of cold on viral infection under certain circumstances. The appropriate circumstances would be when an animal (or man) is infected with a virus, or a particular strain of a virus, that has an optimum multiplication temperature a degree or two lower than the body temperature of the host. Under these circumstances the body temperature of the host could be an important controlling factor in limiting viral multiplication and in keeping the infection a mild, or inapparent, or latent one. And lowering the temperature in the right tissues (surface or internal, depending on the virus) only a degree or two could result in a markedly increased pace of viral multiplication, and thus lead to obvious aggravation or activation of the infection. Under these circumstances we would not expect to see an effect of cold unless the exposure were sufficient to bring about an appropriate drop in temperature in the right tissues. Nor need we expect to see an enhancing effect on a highly virulent virus with a temperature optimum near that of the host tissue. With certain host-virus combinations even an ameliorating effect of cold might be expected if the tissue temperature could be dropped below and maintained below the optimum temperature range of the virus. This concept of an effect of cold due to direct influence on viral multiplication processes within the host cell does not, of course, in itself exclude the possibility that stress, or other physiologic changes due to cold, may contribute to the reaction. But it seems to me that many of the demonstrations of an effect of cold on viral infection can be accounted for on the basis of a direct effect of tissue temperature on intracellular reactions.

WALKER

LITERATURE CITED

1. Bedson, H. S., and K. R. Dumbell. 1961. The effect of temperature on the growth of pox viruses in the chick embryo. *J. Hyg.* 59: 457-469.
2. Boring, W. D., D. M. Angevine, and D. L. Walker. 1955. Factors influencing host-virus interactions. I. A comparison of viral multiplication and histopathology in infant, adult, and cortisone-treated adult mice infected with the Conn.-5 strain of Cocksackie virus. *J. Exp. Med.* 102: 753-766.
3. Boring, W. D., and R. S. Levy. 1962. Studies on the production of B-1 Cocksackie virus by HeLa cells. *J. Immunol.* 88: 394-400.
4. Boring, W. D., G. M. ZuRhein, and D. L. Walker. 1956. Factors influencing host-virus interactions. II. Alteration of Cocksackie virus infection in adult mice by cold. *Proc. Soc. Exp. Biol. Med.* 93: 273-277.
5. Briody, B. A., W. A. Cassel, J. Lytle, and M. Fearing. 1953. Adaptation of influenza virus to mice. I. Genetic and environmental factors affecting an A-prime strain of influenza virus. *Yale J. Biol. Med.* 25: 391-400.
6. Dubes, G. R., and H. A. Wenner. 1957. Virulence of polioviruses in relation to variant characteristics distinguishable on cells in vitro. *Virology* 4: 275-296.
7. Enders, J. F., and H. E. Pearson. 1941. Resistance of chicks to infection with influenza A virus. *Proc. Soc. Exp. Biol. Med.* 48: 143-146.
8. Hoggan, M. D., and B. Roizman. 1959. The effect of the temperature of incubation on the formation and release of herpes simplex virus in infected FL cells. *Virology* 8: 508-524.

TEMPERATURE AND VIRAL INFECTION

9. Kilham, L. 1959. Relation of thermoresistance to virulence among fibroma and myxoma viruses. *Virology* 9: 486-487.
10. Lwoff, A. 1959. Factors influencing the evolution of viral diseases at the cellular level and in the organism. *Bacteriol. Rev.* 23: 109-124.
11. Lwoff, A., and M. Lwoff. 1960. Sur les facteurs du developpement viral et leur rôle dans l'évolution de l'infection. *Ann. Inst. Pasteur* 98: 173-203.
12. Lwoff, A., and M. Lwoff. 1961. Les evenements cycliques du cycle viral. I. Effets de la temperature. *Ann. Inst. Pasteur* 101: 469-504.
13. Marshall, I. D. 1959. The influence of ambient temperature on the course of myxomatosis in rabbits. *J. Hyg.* 57: 484-497.
14. Mykityowycz, R. 1956. The effect of season and mode of transmission on the severity of myxomatosis due to an attenuated strain of the virus. *Austral. J. Exp. Biol. Med. Sci.* 34: 121-132.
15. Sharpe, H. S. 1958. Effect of temperature on the multiplication of foot-and-mouth disease virus in suspensions of kidney cells of the pig. *Nature* 182: 1803-1805.
16. Shope, R. E. 1955. The swine lungworm as a reservoir and intermediate host for swine influenza virus. V. Provocation of influenza by exposure of prepared swine to adverse weather. *J. Exp. Med.* 102: 567-572.
17. Sulkin, E. S. 1945. The effect of environmental temperature on experimental influenza in mice. *J. Immunol.* 51: 291-300.
18. Thompson, R. L. 1938. The influence of temperature upon proliferation of infectious fibroma and infectious myxoma viruses in vivo. *J. Infect. Dis.* 62: 307-312.

WALKER

19. Thompson, R. L., and M.S. Coates, 1942. The effect of temperature upon the growth and survival of myxoma, herpes, and vaccinia viruses in tissue culture. *J. Infect. Dis.* 71: 83-85.
20. Walker, D. L., and W. D. Boring, 1958. Factors influencing host-virus interactions. III. Further studies on the alteration of Coxsackie virus infection in adult mice by environmental temperature. *J. Immunol.* 80: 39-44.
21. Wheeler, C. E., and C. M. Canby, 1959. Effect of temperature on the growth curves of herpes simplex virus in tissue culture. *J. Immunol.* 83: 392-396.

DISCUSSION

CAMPBELL: It seems to me that the enzyme system shown in Figure 1 is an unusually sensitive system as far as kinetics go. If you were to plot the effect of temperature on your enzyme reaction, what would you find?

METCALF: We have done this repeatedly; I would say possibly 30 or 40 times.

CAMPBELL: I am not questioning the results. It's just so unusual. Earlier this afternoon somebody talked about the sensitivity of viruses to temperature as far as susceptibility goes, so this is a rather unusual reaction. Didn't you notice that the effect of temperature between 37° C and 20° C is really a tremendous difference in the activity of your enzyme?

METCALF: In other words, Dr. Campbell, you think that this is more than would seem to be indicated by experience with other systems?

CAMPBELL: Well, it's a little different, I would think. It would be really interesting to do a kinetic study on it.

TEMPERATURE AND VIRAL INFECTION

METCALF: All I can say is, while we haven't performed precise kinetic studies, we have examined the reaction over a period extending from zero hours through 24 hours. We find that there is a linear release of neuraminic acid for twenty to thirty minutes, after which the rate of release slows down greatly, but does continue for eighteen to twenty-four hours. The initial effect of virus dilution upon the release of neuraminic acid is overcome at the end of twenty-four hours. The effect of temperature seems to consist of a retardation of the reaction with less neuraminic acid split off.

CAMPBELL: That will be an interesting system to study thermodynamically.

WALKER: I have been trying to relate this to some work that Dr. Billie Padgett and I have done. Dr. Padgett approached the question of the role of the enzyme of influenza a little differently, and actually aimed at selecting a strain of influenza B virus that was different in its enzymatic characteristics. She was able to get a line of virus that is quite different in its enzymatic characteristics from the parent strain in that its peak zone of activity is at about 35° C, whereas the parent strain has its peak activity at about 37° C. The enzymatic activity of this virus is markedly inhibited at temperatures above 35° C, particularly if calcium ion is removed from the medium. She has made an effort to use temperature as a means of cutting the multiplication cycle and seeing where the effect of this enzyme may be. The parent and variant viruses appear to be very similar in all other respects, antigenic make-up, general characteristics, and so on, except for their temperature optimum for enzymatic activity. She finds that the time at which elevation of temperature will affect the multiplication cycle of the variant virus is only toward the end of the cycle, suggesting again that the enzyme is not particularly important in penetration. Elevation of temperature with this variant virus early in the cycle doesn't have much effect, but late in the cycle it does.

We also have followed the appearance of enzyme and virus in the cell, but we have interpreted the results a little differently. I am a little reluctant to think that the enzyme necessarily has an

WALKER

effect upon the cell. Our interpretation has been that as multiplication goes on and virus is produced possessing enzymatic activity, you see then the appearance of enzymatic activity in the cell. This is associated with cell damage, but it would be difficult to decide which is cause and which is effect.

METCALF: Well, this is perhaps true, but in our case, every time that we demonstrate enzymic competence and show this by enzyme fabrication, we experience a corresponding reduction in the exhibitor content and there is demonstrable damage to the cells. I agree that it is difficult to separate cause and effect, but enzyme fabrication and cell damage are intimately related. Perhaps this would be a compromise between the two viewpoints.

WALKER: Schlesinger, too, has shown cyclic waves of virus level and substrate levels in the cells.

ANDREWES: Dr. Sulkin, you were quoted by Dr. Metcalf. Have you got any comments to make?

SULKIN: No, not particularly, because when those experiments were done back in 1941 and 1942, we knew nothing about neuraminidase, inhibitors, and so forth. It was sort of a naive experiment.

ANDREWES: We were all naive in those days.

SULKIN: The results have been subsequently duplicated by other workers.

CAMPBELL: Frank Lanni has done quite a bit on this inhibitor. Didn't he come up with a chemical nature of it and do some kinetic studies?

METCALF: Lanni¹ studied the interaction between enzymatically active swine influenza and egg white inhibitory mucoprotein. He described the kinetics of inhibitor inactivation, basing his

¹ Lanni, F., and Y. T. Lanni, 1955, *Virology* 1: 40-57.

TEMPERATURE AND VIRAL INFECTION

interpretation of the data upon Gottschalk's model of the structure of urinary mucoprotein.^{2,3} The inhibitor reduction method of assay was used in these studies. There was no attempt to define the chemical structure of inhibitor.

² Gottschalk, A. 1952, Nature 170: 662-663.

³ Gottschalk, A. 1954. The Blakiston Co., New York.

THE INFLUENCE OF COLD ON VIRUS INFECTIVITY

Dr. T. G. Metcalf

Department of Bacteriology
University of New Hampshire
Durham, New Hampshire

ABSTRACT

This study was on the characteristics of influenza A₂ strains which might play a role in facilitating virus invasion of host cells, and the effect of cold upon the invasive process. Strains of influenza A₂ selected for study were isolated from fatal cases of influenza in humans. The strains were examined for mouse toxicity following intravenous injection, cytotoxicity in HeLa and L-cells, mouse and chick embryo ID₅₀ values, and neuraminidase activity. Virus enzyme action was singled out for special attention on the basis of its constant association with A₂ strains. The enzyme activity was determined (1) by means of thiobarbituric acid analysis for free neuraminic acid, and (2) by reduction of the hemagglutination titer of mucoid inhibitor. Substrates used included neuraminmucoid from edible birds nest; neuraminlactose from bovine colostrum, and ovomucin from hens eggs. The rate and extent of enzyme action exhibited by virus showed a progressive decline as the temperature was lowered from 37° C to 4° C. A comparison of enzyme activity, virus titer, and inhibitor concentration in chick embryos at 37° C and 20° C showed corresponding decreases in enzyme activity and virus titer while inhibitor remained virtually unchanged at lower temperatures. Infection of mice was followed by extensive lung damage and death at 4° C. Considerably less damage and fewer fatalities within the test period were found at 20° C. Contrary to the chick embryo experience, the enzyme activity and virus titer showed increases at the lower temperature. Cell monolayers of monkey or hamster kidney were used in conjunction with fluorochrome analysis to follow the course of cell invasion by virus at 37° C and 20° C. Virus was first demonstrated in the cytoplasm around the nuclear membrane at 6 hours following incubation at 37° C. Virus presence in the cytoplasm was shown for at least 72 hours. The course of invasion at 20° C was different. Virus was first detected after 10 hours. It appeared in the cytoplasm, but failed to show a significant increase in numbers. It was possible to show virus accumulating at the periphery of allantoic membranes within 12 hours when embryos were incubated at 37° C. No virus accumulation was observed in membranes from embryos incubated at 20° C. Single-caged mice pre-exposed and maintained at 4° C showed massive virus invasion of lung and bronchi, while group-caged mice showed only minimal virus invasion.

The effect of cold upon the course of influenza virus infections represents a special problem in host-parasite relationships. Is the primary influence of temperature a factor altering the biology of a host, or is it directed against the virus? Which is of greater impor-

METCALF

tance, physiological patterns of the host, or virus infectivity?

This study was concerned with the initial stage of virus-cell interaction using influenza virus with selected hosts as the experimental model. The influence of low temperature upon the interaction has been examined from the standpoint of its effect upon virus infectivity.

The proposal that neuraminidase facilitates the penetration of a host cell by influenza virus presumes that infectious virus should possess enzyme activity. This viewpoint has been presented by Gottschalk (1957) who believes enzyme action renders cell wall mucoproteins more permeable to virus and thereby facilitates virus penetration of a host cell.

This study began with the knowledge that experimental influenza infections have been represented as occurring in the absence of enzyme action (Fazekas, 1948; Fazekas and Graham, 1949). Without adopting a position on the essentiality of enzyme to the infectious process, it was reasoned that clues to the influence of cold upon virus infectivity might be gained by a consideration of the effect of cold upon neuraminidase production by influenza virus.

THE EFFECT OF COLD UPON THE NEURAMINIDASE ACTIVITY OF INFLUENZA VIRUS

Enzyme action was measured by two methods. The thiobarbituric acid (TBA) method of Warren (1959) was used to determine the free neuraminic acid resulting from combination of virus and a neuraminic acid containing substrate. A neuraminmucoid substrate was prepared from oriental "edible birds nest" by a modification of the method of Lawton et al. (1956). Edible birds nest is a salivary mucoid produced by the *Collocalia* species of swift. The second method included the use of ovomucin obtained from hens eggs (Gottschalk and Lind, 1949) in an inhibitor reduction (IR) titration following combination of virus and ovomucin (Isaacs and Edney, 1950).

COLD AND VIRUS INFECTIVITY

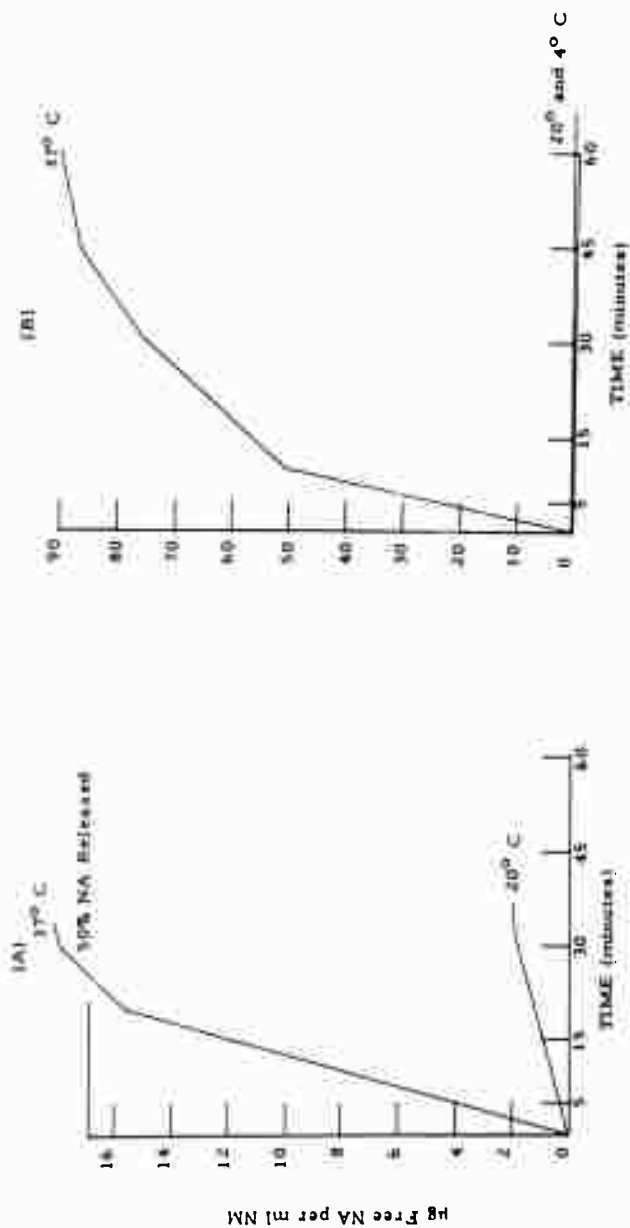


Figure 1a. The release of neuraminic acid from mixtures of influenza virus (A2/Jap 30S/57) and neuramin mucoid at 37°C, 20°C, and 4°C.

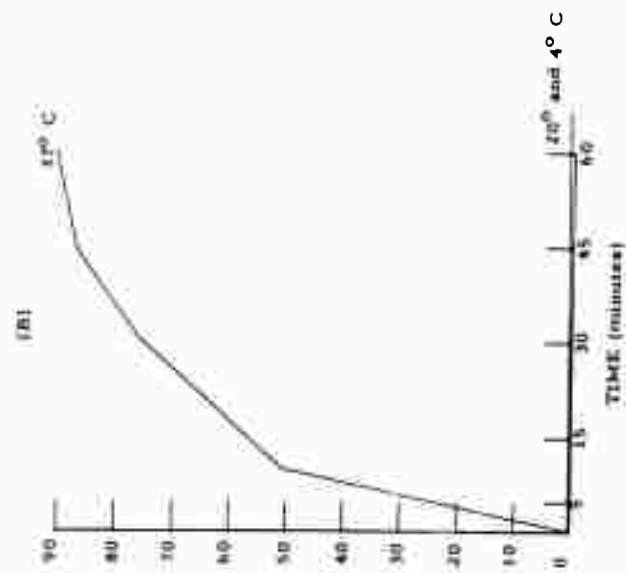


Figure 1b. The per cent split product from enzymic action of influenza virus (A2/Jap 30S/57) on neuramin mucoid at 37°C, 20°C, and 4°C.

METCALF

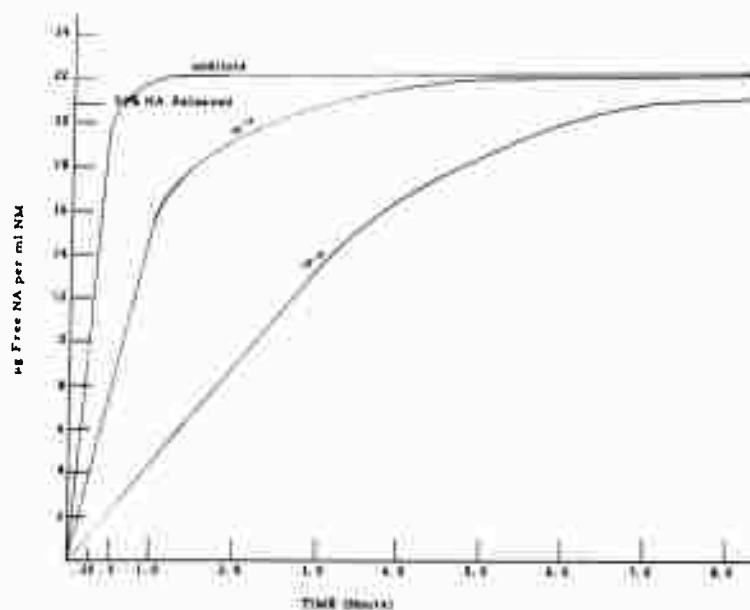


Figure 2. The effect of neuraminidase concentration upon the release of neuraminic acid from mixtures of influenza virus (A2/Jap 305/57) and neuramin mucoid at 37° C.

Using neuraminmucoid and enzyme mixtures incubated at 37° C, free neuraminic acid was linearly split off during the first 20 minutes. When the same mixtures were combined and incubated at 20° C a slow linear increase in free neuraminic acid was observed during the 30 minute test interval. No reaction took place within 30 minutes at 4° C. The use of the IR method for measurement of enzyme action confirmed the findings of the TBA analyses. Differences observed in the results obtained by the two methods were indicative either of a different order of sensitivity, or a difference in the substance being measured. For example the IR titration gave a yield of approximately 90 per cent split product compared to about 53 per cent for the TBA method. Enzymic action at 20° C demonstrated by TBA analysis was not shown by the IR method.

The concentration of enzyme affected the release of neuraminic acid as shown in Figure 2. Not only was the rate of release proportional to enzymic concentration, but also to the total amount released. The same result using other substrate materials at 37° C

COLD AND VIRUS INFECTIVITY

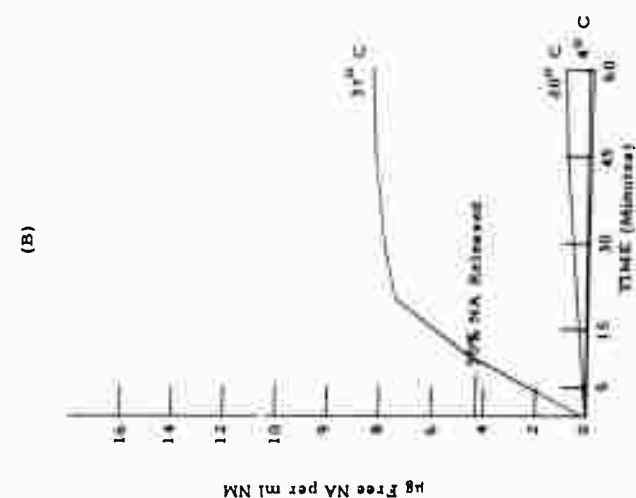


Figure 3b. The effect of temperature upon the enzymic action of influenza virus (B2/Jap 305/57) on ovomucin measured by thiobarbituric acid method.

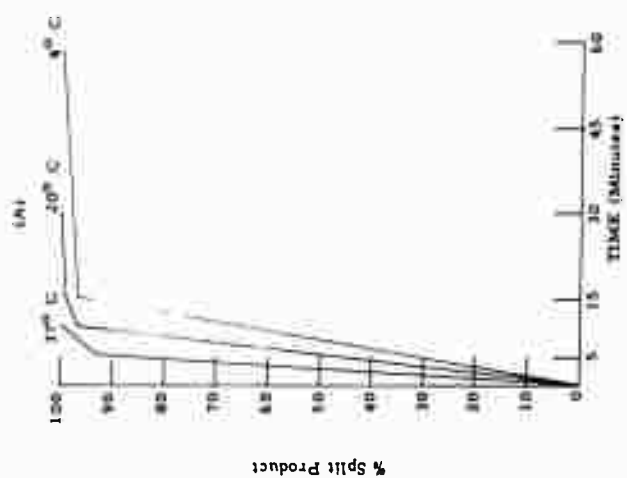


Figure 3a. The effect of temperature upon the enzymic action of influenza virus (B2/Jap 305/57) on ovomucin measured by inhibitor reduction.

METCALF

was reported recently by Noll, Aeyagi and Orlando (1961) and Mayren et al. (1961).

When ovomucin was used in place of neuraminmucoid, different results were obtained. Enzymic destruction of substrate occurred rapidly at all temperatures when measured by the IR method. Only slight differences in the rate of substrate hydrolysis and the time required to reach the same endpoint could be shown. The results of the TBA analyses showed the rate of enzymic attack on ovomucin to be similar to the values found with neuraminmucoid. The efficiency of the hydrolysis was considerably improved, however, and indicated a more labile, more available enzyme-vulnerable structure (Fig. 3).

The inhibitory titer of the two inhibitor preparations for indicator virus varied markedly. Based on the bound neuraminic acid content, ovomucin containing 3×10^{-4} micrograms was inhibitory. Using the same basis neuraminmucoid containing 7×10^{-8} micrograms was inhibitory. The ovomucin inhibitory value of 3×10^{-4} micrograms agreed well with the value $4-8 \times 10^{-4}$ micrograms reported by Gottschalk and Lind (1949) for their preparation of ovomucin. Tamm and Horsfall (1952) reported an inhibitor titer of 3×10^{-4} for urinary mucoprotein at equilibrium with influenza virus. There are no values published for neuraminmucoid.

Ovomucin contained 0.18 per cent total protein, determined by the Biuret test; 0.05 per cent hexose, determined by the modified Orcinol method of Rosevear and Smith (1961); and 1.5 micrograms per ml of total bound neuraminic acid. The neuraminmucoid preparation contained 4.86 per cent total solids, 1.68 per cent total protein, 0.97 per cent hexose, and 11 micrograms per ml of bound neuraminic acid.

For the purposes of the study, the results showed that neuraminidase continued to attack substrate at 20° C, but with decreased efficiency. Both the rate and extent of enzyme action were affected. The marked difference in the values obtained with ovomucin and neuraminmucoid substrates was interpreted as the result of chemically different compounds possessing enzyme-labile structures of varying accessibility and possibly different chemical composition.

COLD AND VIRUS INFECTIVITY

SIMULTANEOUS MEASUREMENTS OF VIRUS, INHIBITOR, AND ENZYME LEVELS DURING INFLUENZA INFECTIONS

In order to examine the effect of low temperature upon virus infectivity, studies were directed toward the relationships existing between virus multiplication, enzyme production, and inhibitor levels in the infected host. The relationships were determined first at the normal environmental temperature of the host and secondly at subnormal temperatures. Both the chick embryo and white mouse were used as experimental hosts. Special attention was given to A2 strains, several of which had been isolated from fatal human cases of influenza.¹ In addition, A and A1 strains were used. The enzyme activity of the different strains varied from the A1 which possessed low reactivity, to the A strains which varied from low to intermediate reactivity, to the A2 strains which had the greatest activity.

A typical experiment using the chick embryo was conducted as follows. Ten-twelve day embryos were injected via the allantoic fluid with 0.1 ml volumes of virus of known concentration. A similar number of control embryos were injected in the same way with the same volume of diluent. Control and test embryos were incubated at 36° C to 37° C and 20° C. Six to ten embryos from each group were removed at regular intervals following injection, the allantoic membranes were harvested, washed, and weighed, and 50 per cent suspensions were then prepared. Aliquots of the suspensions were rapidly frozen and stored at -70° C. The membrane suspensions were examined for their virus content by means of chick embryo LD₅₀ titrations. The enzyme content was determined by TBA or IR assay, and the inhibitor level was measured using indicator virus.

The result of a typical experiment conducted at 37° C using A2 strains was as follows. Virus multiplication was indicated by a steady increase in detectable virus after 1 hour. Beginning at 4 hours the inhibitor content of the membranes began to decline and con-

¹ Obtained through the courtesy of Dr. R. Q. Robinson, International Influenza Center for the Americas, Communicable Disease Center, US Public Health Service, Atlanta, Georgia, and Dr. A. F. Rasmussen, Jr., University of California Medical Center, Los Angeles, California.

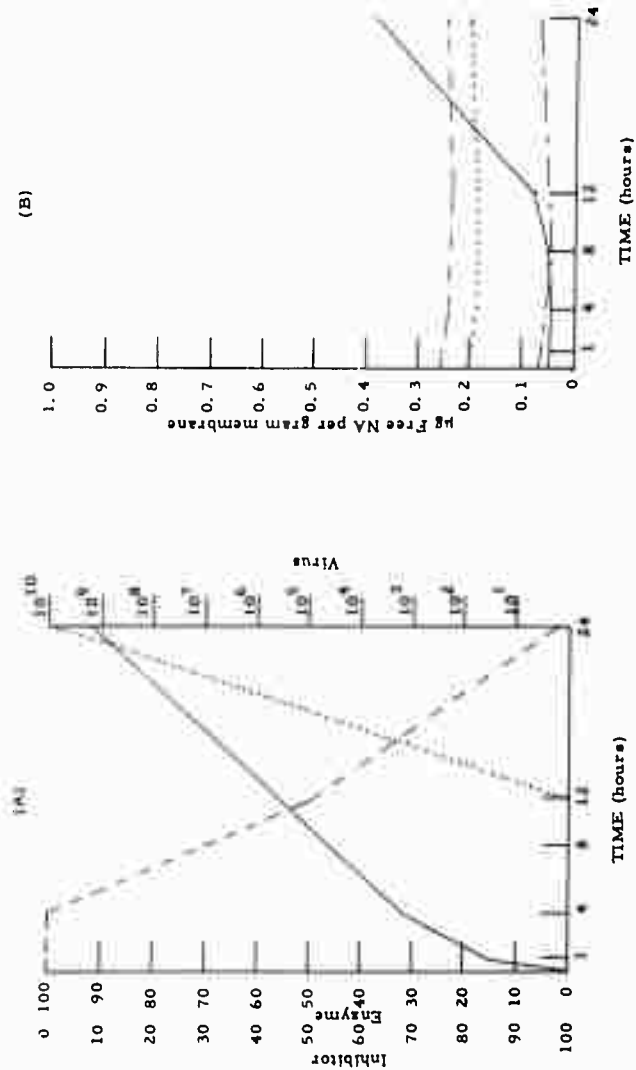


Figure 4a. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Jap 305/57) infected membranes at 37°C. Enzyme production (% split product); ---- Inhibitor content (% reduction inhibitor); ... Virus concentration (log CEID₅₀).

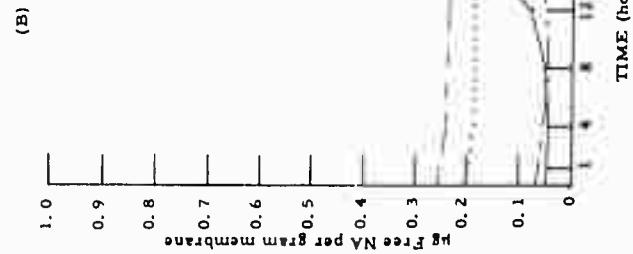


Figure 4b. Enzyme synthesis in influenza (A2/Jap 305/57) infected membranes at 37°C. Control; ---- AF - test; ... AF - control.

COLD AND VIRUS INFECTIVITY

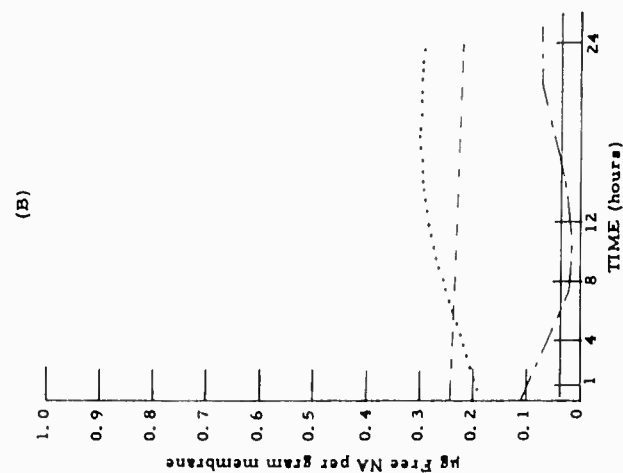


Figure 5b. Enzyme synthesis in influenza A (A2/Jap/57) infected membranes at 20°C. Allantoic membrane (AM) - test; - - - AM - Control; ----- Allantoic fluid (AF) - test; AF - Control.

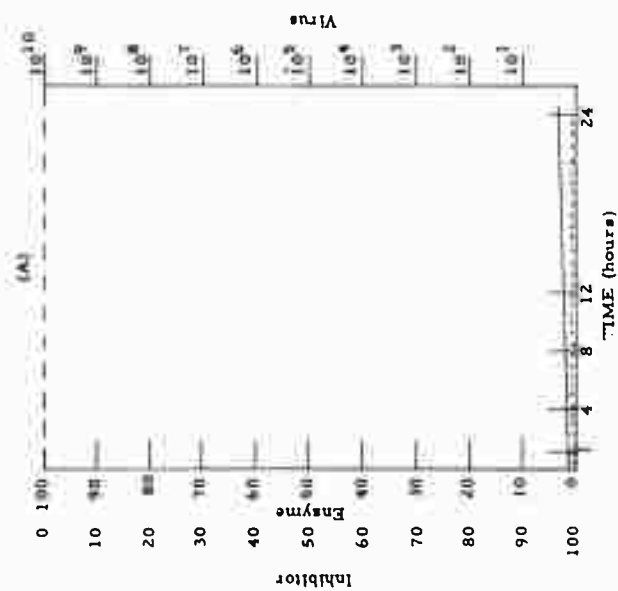


Figure 5a. Virus multiplication, enzyme production and inhibitor content in influenza A (A2/Jap 305/57) membranes at 20°C. Virus concentration (log CEI/50); ----- Inhibitor content (% reduction inhibitor); Enzyme production (% split product).

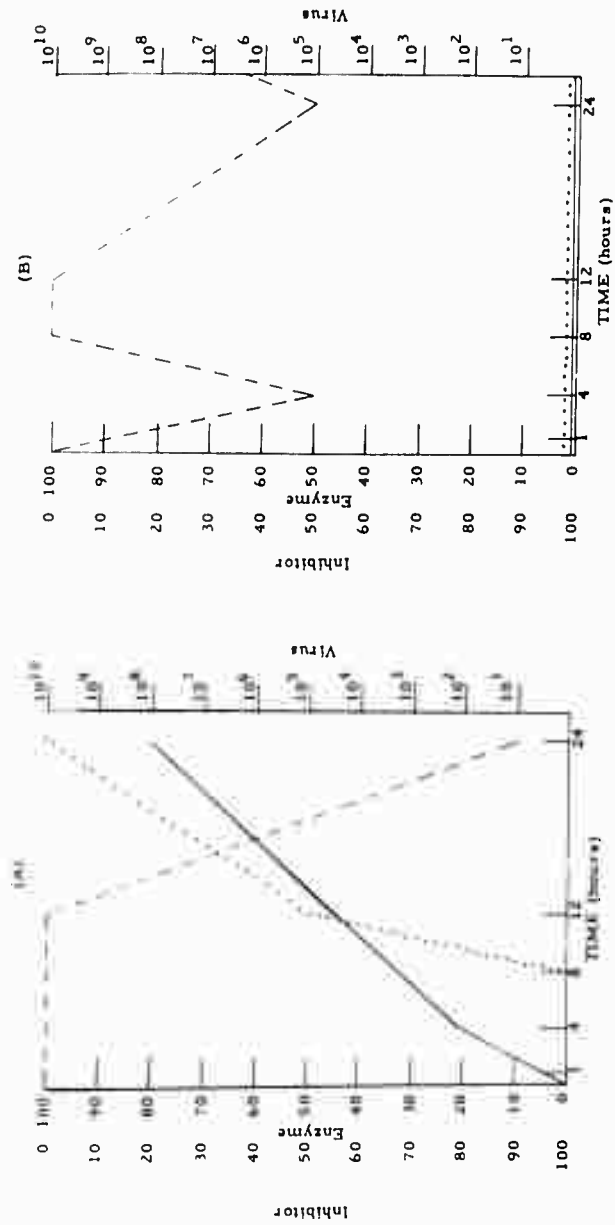


Figure 6a. Virus multiplication, enzyme production and inhibitor content in influenza (A/PR-8/34) infected membranes at 37°C. Virus concentration (log CEID₅₀); ---- Inhibitor content (% reduction inhibitor); Enzyme production (% split product).

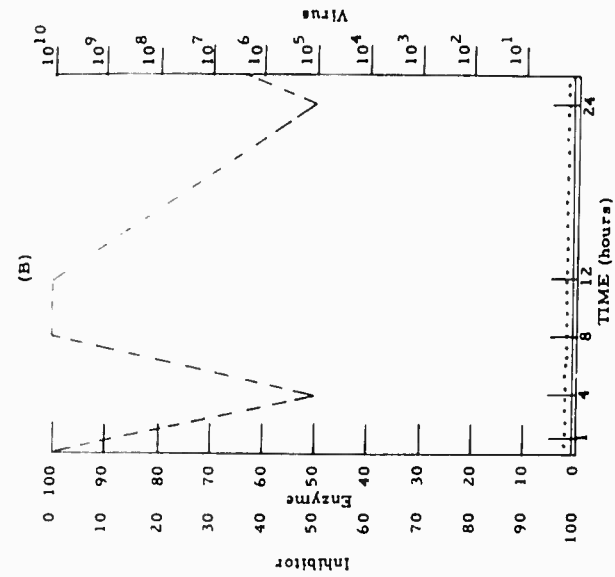


Figure 6b. Virus multiplication, enzyme production and inhibitor content in influenza (A/PR-8/34) infected membranes at 20°C.

COLD AND VIRUS INFECTIVITY

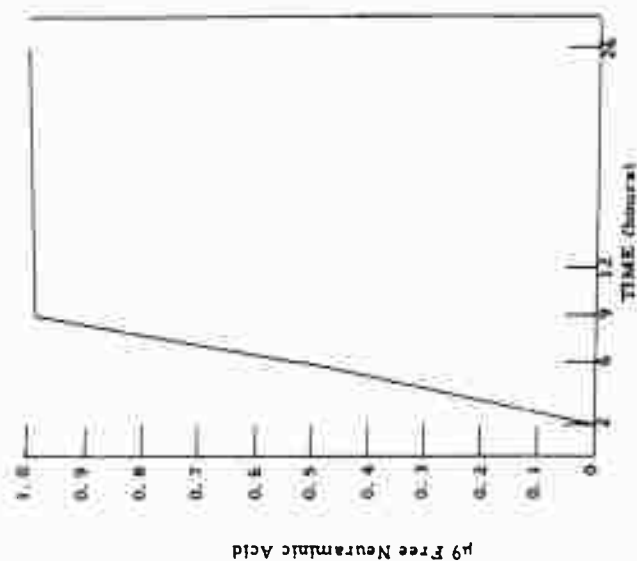


Figure 7b. Neuraminidase synthesis in influenza (A2/Jap 305/57) infected membranes at 37°C. --- Virus concentration (log CEID₅₀); ----- Inhibitor content (% reduction inhibitor); Enzyme production (% split product).

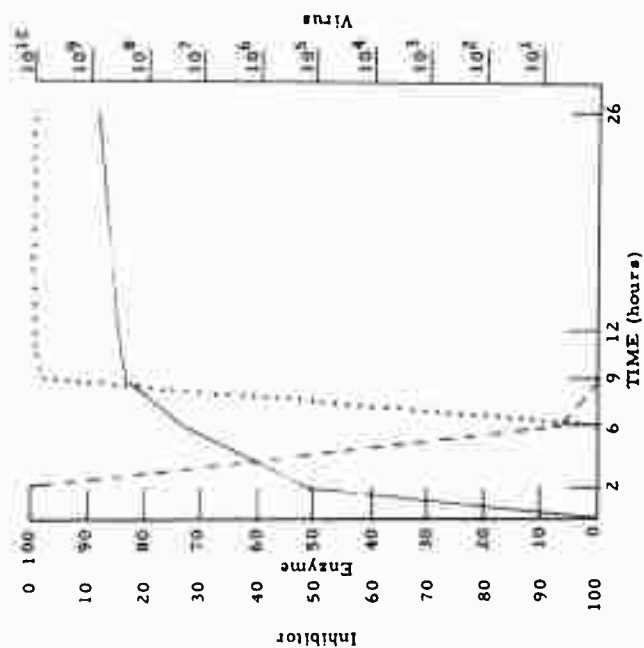


Figure 7a. Virus multiplication, enzyme production and inhibitor content in influenza (A/Pitt-8/34) infected membranes at 37°C.

METCALF

tinued to fall throughout the 24 hour test period. Sometime after 12 hours the production of enzyme within the membranes was detected, with a steady increase shown during the remainder of the test period. Confirmation of the fabrication of enzyme within the infected allantoic membrane was obtained in separate experiments by detection of a rise of free neuraminic acid as measured by the TBA assay. The level of free neuraminic acid in infected membranes began to increase at 12 hours after infection and continued to increase through 24 hours. No increase could be shown for the non-infected membranes (Fig. 4).

When experiments of this kind were conducted with A2 strains in chick embryos incubated at 20° C, the following results were found. There was no significant increase in the virus concentration of membranes, no decrease in inhibitor level, and no enzyme formation was detected. Again there was agreement between inhibitor reduction titrations and TBA assays (Fig. 5).

The findings obtained in chick embryos with A2 strains possessing a high enzyme activity were next compared to the results found with a PR-8 strain of low enzyme activity. Virus multiplication and enzyme production occurred, and the inhibitor level decreased accordingly when the experiment was conducted at 37° C. At 20° C no virus growth or enzyme production was detected. The inhibitor level fluctuated but did not show a steady decline (Fig. 6).

Throughout the study a virus inoculum of 1000 LD₅₀ doses was used. It was decided to determine the effect of increasing the doses given in the inoculum upon the relationships of enzyme production and inhibitor content. Accordingly, an inoculum of 10⁷ LD₅₀ doses per embryo was used. The net result was an accelerated appearance of enzyme and earlier decline of inhibitor. The virus content of membrane reached an early peak accompanied by a rapid rise in enzyme production between 6 and 9 hours. Membrane inhibitor fell rapidly between 2 and 6 hours (Fig. 7).

The pattern of events emerging from these experiments confirmed the existence of a direct relationship between virus multiplication and the appearance of enzyme activity in the allantoic membrane.

COLD AND VIRUS INFECTIVITY

Nell et al., (1961) reported similar findings in an earlier publication which dealt with infected chick embryos. Accompanying the rise in virus titer and enzyme content was a corresponding drop in the concentration of membrane inhibitor. This finding tended to support the contention of Isaacs and Edney (1950) who indicated a role for intracellular receptors in influenza infections on the basis of a correlation between the effectiveness of indicator viruses as interfering agents and their position in the "inhibitor gradient" of allantoic membrane extracts. The time of appearance of enzyme depended on the speed of virus growth. When 10^3 LD₅₀ doses were given, enzyme formation was usually detected after 12 hours. If the number of LD₅₀ doses administered was increased to 10^7 , enzyme formation was observed between 6 and 9 hours. These results were in agreement with previously reported findings on virus multiplication by Henle (1953) and Ackermann and Francis (1954). When the environmental temperature was decreased to 20° C, virus growth failed to materialize, no enzyme was fabricated and the inhibitor level remained unchanged.

Once the relationships of enzyme fabrication, virus growth, and inhibitor decline had been shown in embryos, attention was directed to these same considerations in influenza infected mice. Albino mice were infected intranasally with mouse adapted A2 and A1 strains.² Two groups of mice were established, one group caged at the normal animal room temperature of 20° C, and the other at 4° C. The mice placed at 4° C were individually caged in plastic containers without bedding material. Water was added to the containers in amounts adequate to give a thin discontinuous film of moisture covering the floor. The mice were maintained at 4° C until signs of distress were noted. These included shivering, ruffled fur, apathy to stimuli, and blanched ears. Mice from the two groups received an inoculum previously determined to give 50 per cent lung consolidation at 48 hours in mice maintained at 20° C. An equal number of control mice from the two groups received an inoculum of sterile saline. Further control mice were inoculated to determine the 50 per cent lung consolidation endpoint. Mice from test and control groups at 20° C and 4° C were sacrificed at 0, 8, 24 and 48 hours. Lungs and trachea were examined for their virus titer, enzyme, and inhibitor content.

² Obtained through the courtesy of Dr. T. Francis, Jr., School of Public Health, University of Michigan, Ann Arbor, Michigan.

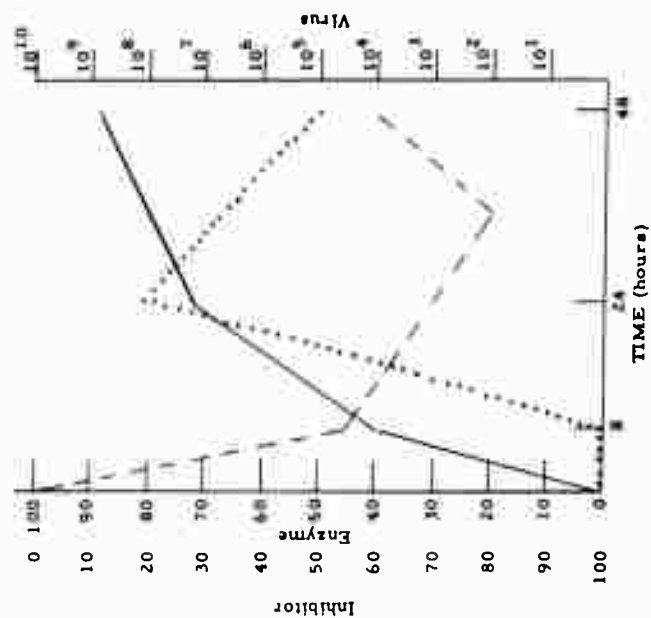


Figure 8b. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Ann Arbor/58) infected mouse lungs at 4°C. --- Inhibitor content (% reduction inhibitor); . . . Enzyme production (% split product).

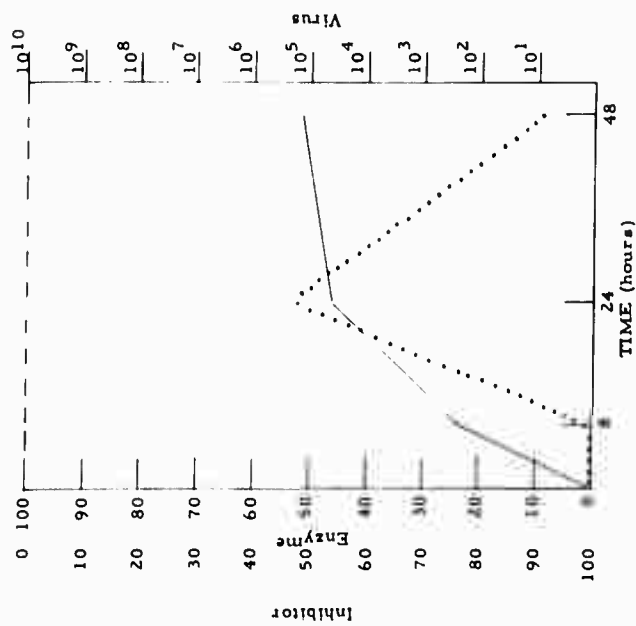


Figure 8a. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Ann Arbor/58) infected mouse lungs at 20°C.

COLD AND VIRUS INFECTIVITY

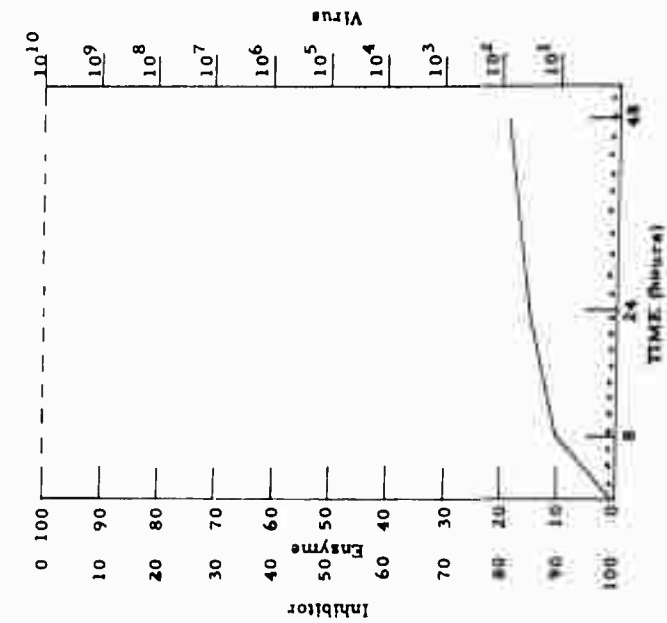


Figure 9a. Virus multiplication, enzyme production and inhibitor content in influenza A/FM-1/47 infected mouse lungs at 20°C. —, Virus concentration; ---, Enzyme content; ···, Inhibitor content.

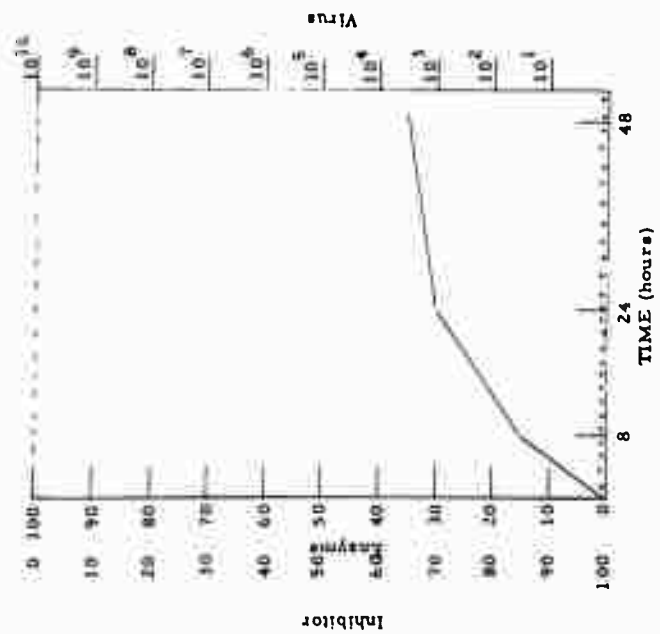


Figure 9b. Virus multiplication, enzyme production and inhibitor content in influenza A/FM-8/24 infected mouse lungs at 20°C. —, Virus concentration; ---, Enzyme content; ···, Inhibitor content.

METCALF

Gross macroscopic examination of the lungs showed complete consolidation to have occurred in the mice kept at 4° C while 50 per cent consolidation was found in lungs removed from mice kept at 20° C. Enzyme production occurred in both groups, but inhibitor levels declined only in the cold exposed mice. A cold environment, therefore, led to a more rapid growth of virus which in turn led to a greater yield of enzyme and a decline in inhibitor content (Fig. 8).

The importance of neuraminidase activity to cell damage was shown by the results obtained following inoculation of mice with A1 and A strains having no detectable enzyme action. Virus multiplication was limited, no enzyme was produced and there was no loss of inhibitor. The lungs removed at sacrifice were normal in appearance and no cell damage could be demonstrated (Fig. 9).

FLUOROCHROME STUDIES OF EXPERIMENTAL INFLUENZA INFECTIONS

Host response to virus infection at the cellular level was studied with the help of the fluorochrome acridine orange. Monkey kidney monolayers were infected with A2 strains of high and low enzyme activity at environmental temperatures of 20° C and 37° C, and examined at intervals up to 5 days. "Flying coverslips" were fixed in chilled absolute methyl alcohol, hydrated, and stained 5 to 9 minutes with a 1 to 10,000 dilution of acridine orange at a pH of 3.8. The coverslips were examined with a Fluorestar microscope used in conjunction with an Osram HBO-200 illuminator. The exciter filter used was a Corning No. 5860, half-thickness filter. Photomicrographs were made using Ektachrome, type B film.

Non-infected monolayers showed the green fluorescing nuclei characteristic of DNA material, with yellowish-white nucleoli. The cytoplasm exhibited a rust color symptomatic of RNA. Six hours after infection the cytoplasm around the nucleus began to show a brighter rust fluorescence. By 18 hours the fluorescence became a bright orange-red color and could easily be distinguished. At 30

COLD AND VIRUS INFECTIVITY

hours cytoplasmic inclusions were observed. From 72 to 96 hours the infected cell showed extensive cytopathogenic change. The specificity of the stain was controlled by the use of RN'ase which made it possible to distinguish the presence of nonspecific staining.

The sequence of events in the invasion of cell monolayers did not differ noticeably at 37°C or 20°C. The presence of virus, its intracytoplasmic growth, cytopathology, and cell destruction, could not be differentiated with certainty at the two temperatures.

Examination of allantoic membranes was accomplished by means of paraffin sections prepared after the method of Coons and Sainte-Marie (1960). Three to five micron sections were cut on a rotary microtome and stained with acridine orange. Virus growth at 37°C was shown to occur predominantly in the entodermal cells lining the allantoic cavity, although some virus staining material was found in the ectoderm. Infected membranes kept at 20°C showed practically no virus staining material in the entodermal cells.

When sections of mouse lung were examined, the extent of virus invasion was shown. A section of non-infected mouse lung failed to show virus-staining material. Sections of lungs from mice infected at 20°C showed virus-staining material gathered around the bronchi as well as scattered throughout the lung. Sections from infected mice of the 4°C group showed a more massive accumulation of virus-staining material. Greater concentrations of virus appeared immediately adjacent to the bronchi.

The results of the fluorochrome examinations furnished visual evidence that the neuraminidase-active strains of influenza used in the study found their way into host cells of chick embryos, monkey kidney monolayers and mice. The initial stage of cell invasion was not affected by the degree of enzyme activity associated with the A2 strains. Thus, strains of high enzyme activity could not be shown to possess a penetration advantage over strains of low enzyme activity.

METCALF

DISCUSSION

The measure of infectious virus used in the study was the ability to multiply sufficiently within a host cell to damage it by means of enzymic attack. The direct relationship shown between cell damage and neuraminidase production led to this conclusion. Proceeding on this basis, the results indicated that cold had no effect per se upon the infectious quality of virus. For example, the penetration of a host cell was not affected, and the invitro effect upon neuraminidase activity was minimal. Instead, cold was shown to exert an influence on the host-parasite relationship subsequent to virus penetration. The findings suggested that cold acted to alter some host structure important for defense and accordingly render the cell more vulnerable to enzyme action. It was postulated that the structure affected by cold was mucoprotein in nature. This viewpoint would represent a modification of Gottschalk's enzyme penetration theory (1957). The important difference lies in the relationship of enzyme activity to cell damage in the present study, rather than cell penetration.

The results were an extension of the findings of Sulkin (1945) who reported a more serious infection in cold exposed mice with little change in mortalities. The fatalities occurring in the present study were attributed to a combination of enzymic competence of the virus strains used and physiologic insult sustained by the mice.

The findings of the present study constituted an interesting contrast to the results reported by Sarracino and Soule (1941). These authors suggested that infection depends on the amount and virulence of virus rather than the general resistance of the host. My study indicated that virus possessing enzyme activity was infectious and thus virulent. It also showed that the severity of influenza infections was not the exclusive result of virus virulence, but could be conditioned by cold-induced physiologic alterations within a host.

An attempt at translation of the results of the present study in terms of the "winter factor" of Andrewes (1958) would be presumptuous. The results did suggest, however, that a study of physiologic alterations of mucoprotein structures of a cell exposed to virus

COLD AND VIRUS INFECTIVITY

possessing enzymic competence might provide clues to the nature of the winter factor.

SUMMARY

Infectious influenza virus was considered to be characterized by an enzymic potential capable of causing host cell damage. No effect of cold upon infectious virus per se could be shown. Any influence exerted by cold upon experimental influenza infections was shown to be closely related to a host-induced effect. Host cell damage was believed to follow enzymic action upon muco-protein structures of the cell.

LITERATURE CITED

1. Ackermann, W. Wilbur, and Thomas Francis, Jr. 1954. Characteristics of viral development in isolated animal tissues. *Advances in Virus Research* 2: 81-108.
2. Andrewes, C. H. 1958. Symposium on the aetiology, spread and control of epidemic influenza. A. The epidemiology of epidemic influenza. *J. Royal Soc. Health* 78: 533-536.
3. Coons, A. H., and Guy Sainte-Marie. 1960. Personal Correspondence.
4. Fazekas de St. Groth, S. 1948. Viropexis, mechanism of influenza virus infection. *Nature* 162: 294-295.
5. Fazekas de St. Groth, S., and D. Graham. 1949. Modification of virus receptors by metaperiodate. Infection through modified receptors. *Australian J. Exp. Biol. Med. Sci.* 27: 83-98.

METCALF

6. Gottschalk, A. 1957. Neuraminidase: The specific enzyme of influenza virus and vibrio cholerae. *Biochim, et Biophys. Acta* 23: 645-646.
7. Gottschalk, A., and P. E. Lind. 1949. Ovomucin, a substrate for the enzyme of influenza virus. I. Ovomucin as an inhibitor of hemagglutination by heated lee virus. *Brit. J. Exp. Pathol.* 30: 85-92.
8. Henle, W. 1953. Multiplication of influenza virus in the entodermal cells of the allantois of the chick embryo. *Advances in Virus Research* 1: 141-227.
9. Isaacs, A., and M. Edney. 1950. Interference between inactive and active influenza viruses in the chick embryo. I. Quantitative aspects of interference. *Australian J. Exp. Biol. Med. Sci.* 28: 219-230.
10. Lawton, V., J. V. McLoughlin, and W. T. J. Morgan. 1956. Solubilization of water-insoluble mucopolysaccharides and new method for preparation of blood group substances. *Nature* 178: 740-741.
11. Mayron, L. W., B. Robert, R. J. Winzler, and M. E. Rafelson, Jr. 1961. Studies on the neuraminidase of influenza virus. I. Separation and some properties of the enzyme from Asian and PR-8 strains. *Arch. Biochem. and Biophys.* 92: 475-483.
12. Noll, H., T. Aoyagi, and J. Orlando. 1961. Intracellular synthesis of neuraminidase following infection of chorioallantoic membranes with influenza virus. *Virology* 14: 141-143.
13. Rosevear, J. W., and E. L. Smith. 1961. Glycopeptides. I. Isolation and properties of glycopeptides from a fraction of human gamma-globulin. *J. Biol. Chem.* 236: 425-435.
14. Sarracino, J. B., and M. H. Soule. 1941. Effect of heat, cold, fatigue and alcohol on resistance of mice to human influenza virus. *Proc. Soc. Exp. Biol. Med.* 48: 183-186.

COLD AND VIRUS INFECTIVITY

15. Sulkin, S. E. 1945. The effect of environmental temperature on experimental influenza in mice. *J. Immunol.* 51: 291-300.
16. Tamm, I., and F. L. Horsfall, Jr. 1952. A mucoprotein derived from human urine which reacts with influenza, mumps, and Newcastle disease viruses. *J. Exp. Med.* 95: 71-97.
17. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* 234: 1971-1975.

DISCUSSION

REINHARD: Dr. Walker, what is the temperature of a baby mouse?

WALKER: I don't know, but I would like to. This brings up a very interesting point. I indicated that we have begun work involving using both tissues of baby mice and adult mice. It is quite evident that raising the body temperature of a baby mouse does not produce the same effects that I showed here for the adult mouse. We have exposed infant mice to incubator temperatures up to about 35° C. Above this temperature, the infant mice do not survive. I think perhaps the baby mice could tolerate higher temperatures, but the mother refuses to feed them or her lactation stops at higher temperatures. She apparently has enough troubles at that temperature without having these little furnaces hanging on her, so she retreats to the opposite side of the cage and the mice are not fed. I don't know what the temperature of baby mice is at ambient temperatures of 35° C, but I think it is probably higher than normal. I haven't succeeded, so far, in finding a probe that will allow me to measure the body temperature of a forty-eight-hour-old infant mouse.

One other thing should be pointed out, too, and that is that the pancreas appears to be different from other tissues. Coxsackie virus will multiply in the pancreas of the adult mouse even at

METCALF

ordinary temperatures, but one can raise the body temperature sufficiently so that even this multiplication is choked off.

NUNGESTER: This pancreas thing intrigues me very much.

WALKER: It intrigues me, too, because there are other viruses that seem to multiply well in the pancreas.

NUNGESTER: But by and large, the pancreas is not susceptible to bacterial infection, at least as far as I know.

WALKER: But I think it is susceptible to a number of viruses.

REINHARD: I might offer the information of some recent gastroenterological research which indicates that the pancreas has different proclivity for amino acid uptake. Certain kinds of amino acids are readily absorbed by the pancreas, and this might be one of the differences causing increased tissue susceptibility.

WALKER: So far, we have had great difficulty in trying to get it to persist or multiply in culture.

METCALF: Dr. Walker, I think you have a very interesting theory. In view of your results as well as the work of Dubos and Wenner,¹ I wonder if you are forced to describe a polydisperse virus population with respect to genetic variance. Are you selecting one member of a polydispersed population at a given temperature?

WALKER: I am not any more worried with that than with any other explanation of multiplication at any temperature. I think temperature is going to be a selective force on virus populations, and I think that, as Lwoff has shown, it is possible to isolate strains that are capable of multiplying at higher temperatures. This is part of the basis for his contention that temperature is related to virulence. Is this pertinent to your question?

¹ Dubos, G. R., and H. A. Wenner. *Virology* 4: 275-296.

COLD AND VIRUS INFECTIVITY

METCALF: I wondered if you felt this to be a condition which might prove deleterious to your theory.

WALKER: No, I think that this is reasonable. This is just another selective pressure, and we will have the same problems with selection that will apply to any other explanation of the effect of temperature on infection.

CAMPBELL: I might point out that it is pretty well known that in neonatal animals there is a question whether they produce any antibodies at all so that when you expose them to antigen, they may actually exhibit a so-called tolerance or immune paralysis. Their immune mechanism is not working at this point. You had an exposure of six days at 25° C.

WALKER: And then a shift to cold.

CAMPBELL: Yes, and then in this case, you didn't have any effect.

WALKER: Right.

CAMPBELL: I was wondering whether you thought that this exposure, some way or another, actually causes an adaptation.

WALKER: The point was, if they were held at ordinary room temperature for as long as six days after receiving virus, then the shift to 4° C had no effect, but after only four days, the cold still caused high mortality. My explanation or speculation is this; by six days, the virus has been reduced to very low levels in practically all of the tissues except the pancreas. By that time, there is still a lot of antibody in the blood, and although virus activity might be increased in the pancreas, there would be no opportunity for it to be disseminated widely to other tissues that would be made more susceptible by the lowered temperature. The virus must be widely spread to cells before antibody appears in the blood, and the exposure to cold must occur while there is still a sufficient quantity of virus in the tissues. I would expect in most of these tissues that the virus is pretty well gone by six days.

METCALF

PREVITE: Your findings are parallel in some ways to those that I have reported on endotoxin. If cold exposure was delayed too long, the animal had manipulated this poison somehow so that he was no longer sensitized to it by cold stress.

WALKER: Then your test material is gone.

PREVITE: Correct.

SULKIN: Dr. Campbell called attention to the fact that the infant mouse does not produce antibody. Cocksackie viruses are unique among viruses in that they elicit an antibody response very, very, rapidly. In man, for example, antibodies are demonstrative very early after first signs of infection. Yet a week-old mouse will produce antibody, whereas the one-day-old mouse will not. Overland showed that the maturation of the antibody forming mechanism in the animal accounts for this rapid acquisition of resistance to infection with Cocksackie viruses.

MITCHELL: Will someone please define for me this infant mouse? Don't say baby; I know that.

WALKER: In Cocksackie B-1 virus infection, for instance, the mouse is most sensitive to the virus up to about forty-eight hours in age. Large inocula of this virus will cause lethal infections in mice up to about eight days of age, but then there is a very abrupt cut-off with no deaths occurring in mice inoculated at an older age. But the mice are most susceptible to small inocula in their first forty-eight hours after birth.

MITCHELL: I was interested in those extra days of exposure; after this delay, the animal would be ten, eleven, or twelve days old.

WALKER: No, you misunderstood, because this exposure to cold is in adult mice. The infant mouse enters in here only because the adult mouse in the cold behaves rather like the infant mouse at normal room temperature.

PREVITE: Dr. Walker, in some of your data you mentioned

COLD AND VIRUS INFECTIVITY

that you used ACTH, and this apparently didn't enhance viral multiplication. What was the regimen that you used and how many injections did you give?

WALKER: Four mg in gel, as I recall, given every twelve hours for a period of six days. ACTH has been shown very rarely to have an effect upon viral infections in the mouse. I don't understand why it should not. You showed the effect of ACTH upon toxin, but this may be quite a different thing. ACTH does produce physiological changes in the mouse that indicate that the ACTH is active, but the failure of ACTH to aggravate viral infections seems to be a peculiarity of the mouse. Cortisone has quite an effect upon viral infection in the mouse, but ACTH has very little.

McCLAUGHRAY: Certainly the thesis that Dr. Watson has presented appeals to me very much as a person interested in human physiology because of the fact that the general pattern of interactions of related functions of cells and tissues fits the category. The modifications of a particular kind of cell reaction or tissue reaction modifies the balance among all functions, and therefore, the modification of temperature at which cells are held might very well favor certain enzymic processes which would be involved, perhaps, in replication of viral protein as compared with certain other cellular processes.

MARROW: Does the level of interferon which responds to the increase in temperature account for part of this decrease in virulence with increased temperatures?

ANDREWES: I think it may work another way. Issacs gave a review on interferon at the Montreal meeting; one of the things that he mentioned was that he has been in touch with Lwoff about this, and he feels, concerning the virulence of the strains of virus which do better at high temperatures, that perhaps they are able to do so because they become less sensitive to the action of interferon.

SUPPRESSIVE EFFECT OF LOW ENVIRONMENTAL TEMPERATURE ON VIRAL INFECTION IN BATS¹

S. Edward Sulkin and Rae Allen

Department of Microbiology
Southwestern Medical School
Dallas 35, Texas

ABSTRACT

Having established the susceptibility of insectivorous bats held at 24° C to 29° C to experimental infection with rabies and encephalitis viruses and determined that virus particles introduced peripherally may invade and multiply in brown fat in addition to other tissues, studies were extended to establish the influence of low temperature on the course of these infections in these animals. Viral proliferation in interscapular brown fat, an organized, bi-lobed structure believed to be present in all hibernating animals, suggested that this tissue might provide a focus of infection or target organ from which virus could disseminate to other tissues. In view of the function of brown fat in maintaining the hibernating animal, it seemed logical to assume that virus particles present in this tissue would remain viable during periods of hibernation, sustained by the same mechanism which nurtures the whole animal, and, upon arousal of the animal would multiply and disseminate to other sites. This rationale has formed the basis for our studies on the influence of low temperature on viral multiplication in bats and the data to be presented will be drawn from the following major areas under investigation: (1) The susceptibility of various species of bats maintained at 24° C to 29° C to experimental infection with rabies and encephalitis viruses. (2) The influence of low temperature on initiation of viral infection in bats and on the course of a previously established infection in these animals. (3) The influence of low temperature on antibody formation in bats in response to bacterial and viral antigens (some information relative to rates of antigen and antibody degradation in the torpid bat will also be included). (4) Reference will be made to studies on the influence of low temperature on viral multiplication in monolayer and explant cultures of tissues of the bat as compared with tissues of warm blooded animals.

¹ The original work reported herein was supported by research grants from the Caruth Foundation, the National Institutes of Health, United States Public Health Service, and the Commission on Viral Infections, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army.

SULKIN AND ALLEN

The isolation of rabies virus from naturally infected insectivorous bats in the United States in recent years has focused the attention of epidemiologists and virologists on the Chiroptera as still another reservoir host for this virus in nature. Since our laboratory reported the first human death believed to have been due to the bite of a naturally infected bat (Sulkin and Greve, 1954), we became interested in the association of bats with rabies and more especially in how these animals are able to survive infection with this hitherto presumed fatal viral infection. Investigators in South America and Trinidad demonstrated that the vampire bat could suffer sustained infections with rabies virus and pass the virus through infected saliva to susceptible animals without themselves showing overt symptoms or succumbing to the disease (Enright, 1956). We now have experimental evidence that insectivorous species of bats native to the United States may be infected with rabies virus and never show symptoms of the disease even though virus can be demonstrated in brain as well as other tissues (Sulkin, 1962).

RABIES STUDIES IN INSECTIVOROUS BATS

Studies on the course of experimental rabies infection in insectivorous bats were undertaken with the view to determining the susceptibility of these animals to peripheral inoculation with rabies virus and to ascertain which tissues were involved in the infection (Sulkin et al., 1959). We have also attempted to determine the influence of certain physiological characteristics of the bat on experimental rabies infection in these animals (Sulkin et al., 1960; Sims, Allen and Sulkin, unpublished data). In this regard, the phenomenon of hibernation was the first area to receive attention.

Species of bats which inhabit temperate zones are efficient hibernators who remain quiescent for several months of the year. It has been suggested that this conservation of energy is the reason why these mammals, despite their small size, are relatively long-lived (Griffin, 1958). Also, many investigators (Rasmussen, 1923, 1924; Remillard, 1958; Johansson, 1959, 1960; Kayser, 1961) believe that

VIRAL INFECTION IN BATS

the so-called "hibernating gland", a bilobed, organ-like accumulation of brown adipose tissue found in the interscapular region of hibernating animals (and in certain non-hibernators as well), plays a role in sustaining the hibernating animal during its period of winter dormancy. Histochemical analysis of the interscapular brown fat of the little brown bat (*Myotis l. lucifugus*) indicates that this tissue builds up quantities of lipid during pre-hibernation months and that the various biochemical constituents of this tissue are depleted as hibernation progresses (Remillard, 1958). Studies comparing the nature of brown and white fat of several animal species indicate that brown adipose tissue is histologically distinct and physiologically more active than white fat (Fawcett, 1952). Recognition of the role of brown fat in sustaining the hibernating bat during periods of inactivity, together with reports dealing with the multiplication of Cocksackie virus (Pappenheimer et al., 1950; Grodums and Dempster, 1959) and poliovirus (Shwartzman, 1952) in the brown fat of mice and hamsters, suggested that this tissue of bats might provide a site for rabies virus sequestration in the asymptomatic host; a latent focus of infection from which virus sustained during the period of hibernation by the same mechanism which nurtures the whole animal could be activated to invade and multiply in other tissues, including salivary gland, and thereby be perpetuated in nature by this host. Accordingly, experiments were undertaken to determine whether rabies virus introduced peripherally would, in fact, invade and multiply in the brown adipose tissue of insectivorous bats. Data compiled from such a study (Sulkin et al., 1959) are summarized in Table I. Two species of bats, the Mexican free-tailed bat (*Tadarida b. mexicana*), which is a quasi-hibernator, and the little brown bat (*Myotis l. lucifugus*), a true hibernator, were used in these experiments. A strain of canine rabies virus isolated from the brain of a fatal human case was used. Each bat received an intramuscular injection into the heavy muscle over the chest of approximately 8000 mouse i.c. LD₅₀ contained in 0.1 ml.

Although the Mexican free-tailed bat proved to be relatively unsusceptible to experimental rabies infection, virus was demonstrated in the brown fat of 22 per cent of those animals shown to be infected by viral assay in white Swiss mice. The infection in this species was most evident 20 to 40 days after intramuscular inoculation of virus. On the other hand, rabies virus was found to be widely distributed on the little brown bat 9 to 26 days following inoc-

Bat Species	No. infected* No. tested	Per cent infected	Virus demonstrated in**		
			Brown fat	Salivary gland	Brain
<u>Tadarida b. mexicana</u>	104/492	21.1	22.1	35.6	87.5
<u>Myotis l. lucifugus</u>	54/143	41.3	30.5	16.9	91.5

Table 1. Susceptibility of bats to canine rabies virus (Thompson strain) following intramuscular inoculation. Compiled from: Sulkin, S. E., et al., 1959. Studies on the pathogenesis of rabies in insectivorous bats. I. Role of brown adipose tissue. J. Exp. Med. 110: 369-388. *Virus inoculum approximately 8,000 mouse intracerebral LD₅₀. **Per cent positive among animals shown to be infected.

VIRAL INFECTION IN BATS

ulation, and virus concentration in some of the tissues approached the level of the stock mouse brain virus suspension used in inoculating these animals. Virus was demonstrated in the brown fat of 30 per cent of the experimentally infected *Myotis*. These data, together with the recent demonstration of rabies virus in the brown fat tissue in naturally infected insectivorous bats, would support the hypothesis that this tissue provides nutriment for a latent focus of infection (Bell and Moore, 1960; Sulkin, 1962). Studies are in progress to determine the frequency with which virus can be demonstrated, under natural conditions, in the brown adipose tissue of bats netted in different geographic areas at different times of the year.

The mechanism by which brown fat may actually serve as a depot for storage of rabies virus is still under study. A working hypothesis concerns the possibility that at least in hibernating species, rabies virus sequesters in brown adipose tissue in the quiescent host during the period of hibernation and is subsequently activated by the physiological alterations which occur prior to awakening and by emergence into a warmer environment. Studies concerned with the effect of low environmental temperature on the pathogenesis of rabies in insectivorous bats would suggest that this may actually be the case. The remaining portion of this discussion will be limited to those areas which relate to the subject of this conference.

It is clear from previous discussions in this conference that environmental temperature has been shown to have a significant effect on several experimental virus-host systems, both in vivo and in vitro. It is also apparent that the outcome of such experiments depends on the particular virus used and on the host or cell system under study (Sulkin, 1945; Walker and Boring, 1958; Hoggan and Roizman, 1959; Lwoff, 1959).

During the course of studies on the role of Chiroptera as reservoir hosts for viruses in nature, it became evident that these experiments with bats provided an unusual opportunity for determining the effect of temperature on viral multiplication in the intact animal. The bat does not possess a thermoregulatory mechanism for maintaining a constant normal body temperature, but rather, the body temperature of a bat is that of his environment, except when he is in the active state of walking or flying (Hock, 1951; Morrison, 1959).

SULKIN AND ALLEN

The body temperature of resting bats has been shown to parallel closely that of their environment over a range of 2°C to 30°C , and the metabolic rate of these animals varies directly with their body temperature (Hock, 1958).

Data have been accumulated comparing the course of experimental viral infections in bats held at 5°C or 10°C with groups held at 24°C or 29°C and with groups held at low temperatures for a period of time and then transferred to room temperature (24°C) or 29°C (Sulkin et al., 1960). Animals held in the cold become torpid within hours of being placed at the low temperature, and we have on occasion determined the rectal temperature of a number of cold-adapted bats and found it to be quite close to ambient. On the other hand, we cannot be positive that animals held at 24°C or 29°C maintained constantly body temperatures equivalent to their environment. For a period of time following the handling associated with virus inoculation and transfer to experimental cages, the bats appear excited and are quite active in moving about their cages. It is likely that during this period the animals have body temperatures well above ambient. Within a day or so, however, they become quiet and hang in groups in their cages; they are seldom seen moving about except in the evening when they come down to the floor of the cage to receive the food and water offered daily. There is doubtless considerable variation among body temperatures of individual bats at these higher temperatures, depending on their degree of activity. The difficulties inherent in recording individual body temperatures on large numbers of infected bats precludes the possibility of obtaining such data. In this presentation we will be comparing the course of viral infection in animals whose body temperatures we know to be low (ambient temperature 5°C or 10°C) with the course of the infection in animals whose body temperatures we feel must have fluctuated between ambient (24°C or 29°C) and possibly 36°C or higher (Morrison, 1959).

Early in these studies it became apparent that animals collected during the fall or early winter months survive for longer periods of time in the cold than animals obtained in the spring or summer months. Subsequently, these experiments were initiated only in the fall, even though they could only be repeated or extended on a yearly basis. Furthermore, a recent report by Menaker (1962) indicates that the state of hypothermia achieved with summer bats is not

VIRAL INFECTION IN BATS

identical with their winter hibernation, and serves to emphasize the necessity for carrying out low temperature studies in bats gathered during the fall months.

Table II summarizes a study of the suppressive effect of simulated hibernation on the susceptibility of little brown bats to two strains of rabies virus. Of those receiving the canine rabies virus (Thompson) and placed in the cold room, infection was demonstrated in only 7.5 per cent of 40 bats studied over 40 days. The infection rate among animals kept at 29° C was 36 per cent, and virus was demonstrated between the ninth and thirtieth day following virus inoculation. It is interesting that virus was demonstrated more frequently in the brown adipose tissue (25.7 per cent) than in the salivary gland (11.4 per cent). The infection rate was even higher among animals held in the cold for two weeks and then transferred to the warm room. Again virus was demonstrated more frequently in the brown fat (30.4 per cent) than in the salivary gland (17.1 per cent). Quantitative data not shown in this tabulation indicate that virus titers in the few animals which developed evidence of infection while in simulated hibernation were $10^{-1.5}$ or less, while titers in various tissues of animals in the warm room ranged from one log unit to as high as $10^{-4.5}$. In the experiment with the rabies virus (59 V13B) recovered from the pooled brown adipose tissue of naturally infected little brown bats, virus was again demonstrated infrequently in tissues of animals inoculated and placed directly in the cold room, but remained viable and was activated when animals were transferred to the warm room. Although the infection rates were similar with both rabies virus strains, a careful study of the results revealed differences in the patterns of infection produced by these viruses in active bats and in those awakened following a period in simulated hibernation. The bat rabies virus occurred more frequently in the brown adipose tissue than in either salivary gland or brain. It can be seen that virus was detectable in the interscapular brown fat in 92 per cent of animals shown to develop infection, and in the salivary gland and brain in only 28 per cent and 50 per cent respectively. Rabies infection was demonstrated in 42 per cent of the animals held in the cold for 17 days before transfer to the warm room, and the infection pattern seemed to be affected by the period in simulated hibernation. In these animals virus was demonstrated in the brown fat, salivary gland, and brain of infected animals with about equal frequency.

Rabies virus strain	Inoculum (mouse i.c. LD ₅₀)	Environmental temperature C	No. infected No. tested	Per cent infected	Virus demonstrated in*		
					Brown fat	Salivary gland	Brain
Canine (Thompson)	8,000	5°	3/40	7.5	(1)	(1)	(2)
		29°	35/97	36.1	25.7	11.4	91.4
		5°-29°**	23/47	48.9	30.4	17.1	91.3
Bat (59VI3B)	6,000	5°	2/47	4.3	(1)	0	(1)
		29°	14/28	50.0	92.0	28.5	50.0
		5°-29°**	21/50	42.0	57.1	52.4	66.6

Table II. Suppressive effect of simulated hibernation on the susceptibility of bats (*Myotis l. lucifugus*) to rabies virus following intramuscular inoculation. Data adapted from: Sulkin, S. E., et al., 1960. Studies on the pathogenesis of rabies in insectivorous bats. II. Influence of environmental temperature. J. Exp. Med. 112: 595-617. *Per cent positive among animals shown to be infected; figures in parentheses refer to numbers of animals. **Animals held at 5° C for 14 days before transfer to 29° C. ***Animals held at 5° C for 17 days.

VIRAL INFECTION IN BATS

suggesting that after a period of latency in a dormant animal, activated virus may reach the salivary gland more rapidly and with greater frequency. In addition, quantitative studies have indicated that the virus concentration in this tissue is greater than in infected animals which have not experienced a period of hibernation. These data are presented graphically in Figures 1 and 2.

The results of these experiments suggest that seasonal fluctuations in environmental temperature may provide not only a mechanism for virus storage in this reservoir host, but may also increase the chances of virus transmission by this host. It is recognized that this concept may only apply for strains of rabies virus which circulate in bat populations.

ARTHROPOD-BORNE VIRUS INFECTIONS IN BATS

During the course of studies on experimental rabies infection in insectivorous bats, we became interested in determining how these animals would react to experimental infection with other viral agents. A survey of the literature (Sulkin, 1962) indicated that bats have been associated in various ways with a number of disease-producing agents, suggesting that these animals might act as reservoir hosts for many viruses in nature. An area of particular interest to us, from an epidemiological standpoint, concerns the mechanisms involved in the overwintering of the arthropod-borne viruses. Although investigators have long sought to determine the whereabouts of these viruses during the winter months in temperate zones where mosquito vectors do not carry out year-round transmission cycles, this void still remains in our understanding of the complex biological life cycles of these disease-producing agents. Reports of the susceptibility of bats to experimental infection with various arthropod-borne viruses (Ito and Saito, 1952; Corrigan, LaMotte and Smith, 1956; LaMotte, 1958) suggested this animal as an additional reservoir host, but provided only limited information as to the course of the infection or the tissues involved. We were prompted, therefore, to pursue studies on experimental arbovirus infection in bats to de-

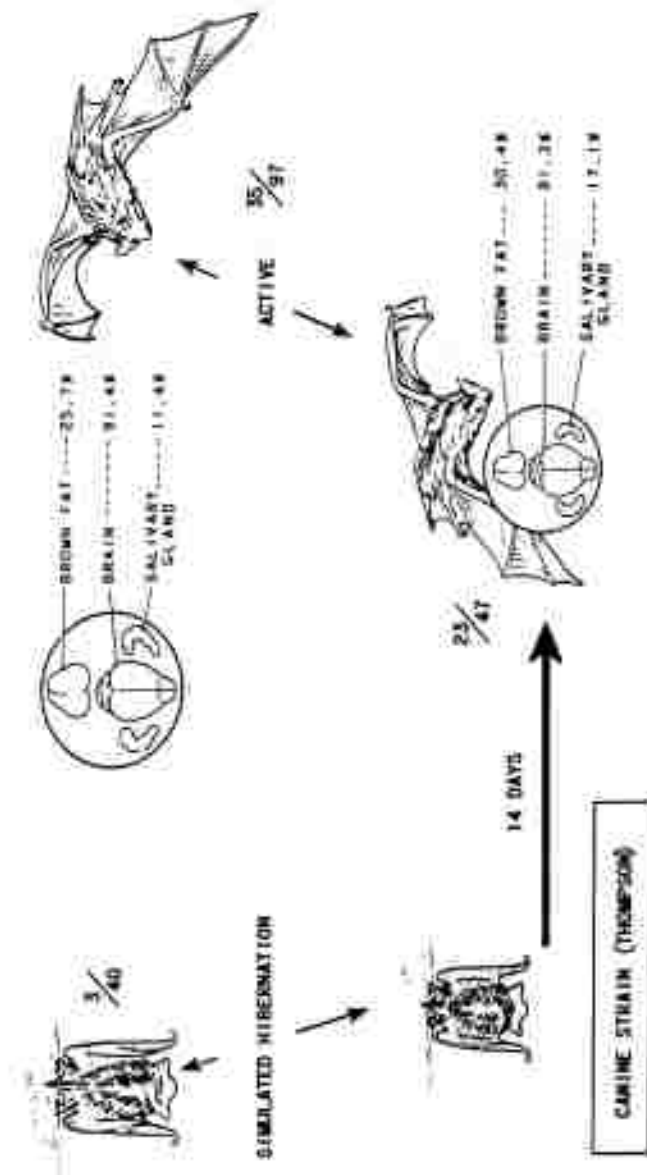


Figure 1. Influence of environmental temperature on frequency of canine rabies virus (Thompson strain) in brown fat, brain, and salivary gland of bats (*Myotis l. lucifugus*) following intramuscular inoculation. Each animal received 8,000 mouse intracerebral LD₅₀. Fractions refer to number infected/number tested. Percentages are based on number of animals shown to be infected.

VIRAL INFECTION IN BATS

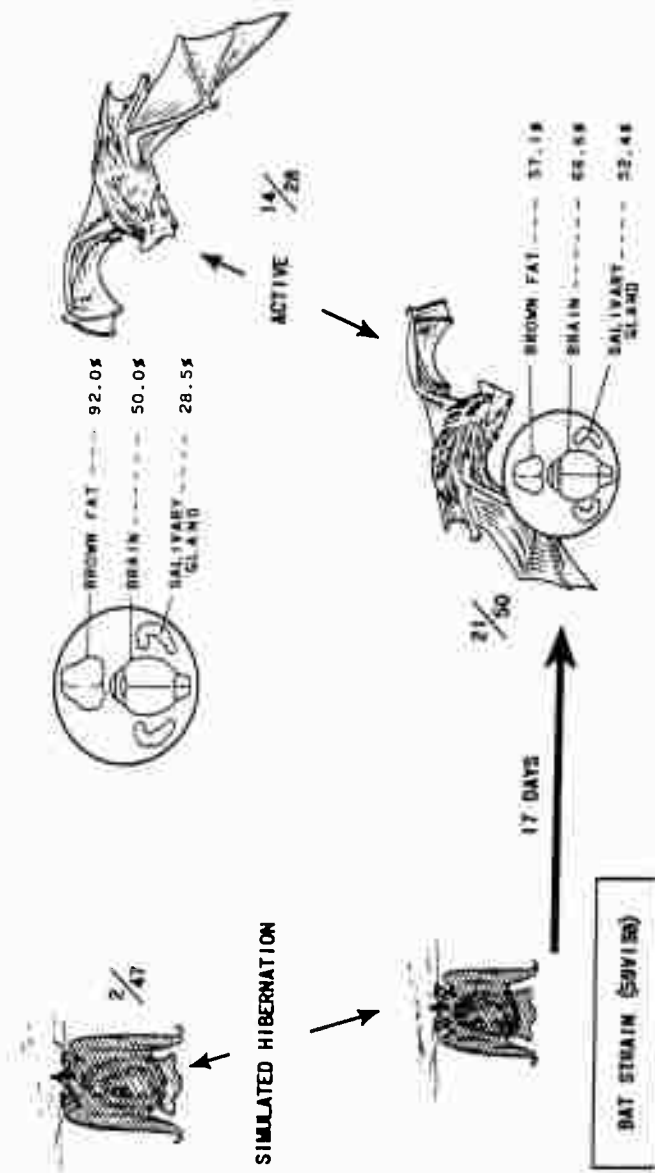


Figure 1. Influence of environmental temperature on frequency of termination of hibernation of bats (Myotis 1, 1949) following intramuscular injection. Each animal received 0.100 mg/ml intramuscular 1.0% solution of virus. Percentages refer to number infected/number tested. Percentages are based on number of animals shown to be infected.

SULKIN AND ALLEN

determine the degree of susceptibility of various species to certain virus strains and to locate the tissues involved in the infection which provided sites for viral proliferation and resultant viremia. Again, emphasis was placed on determining if virus invaded and multiplied in interscapular brown adipose tissue, thereby providing a mechanism for survival of arbovirus particles in the hibernating animal in a manner similar to that described for rabies virus.

In the initial experiments (Sulkin, Allen and Sims, 1960), we were able to confirm certain aspects of the observations reported by Corrigan et al. (1956) and LaMotte (1958). Viremia was demonstrated in bats following peripheral inoculation of Japanese B or St. Louis encephalitis viruses and, in addition, the lipotropic characteristic of these viruses was demonstrated. These experiments have now been extended to include various bat species and several virus strains. Bats were inoculated subcutaneously and placed in specially designed cages which allowed them to receive fresh food and water daily with minimum danger to the caretakers. The virus inoculum consisted of approximately 150 weanling mouse i.c. LD₅₀ of mouse brain suspension, or infected tissue culture fluid, or bat blood. To simulate natural circumstances, bats were kept in near darkness throughout the day and were offered food at the close of each working day. Unless otherwise indicated, animals were maintained at 24° C ± 2° C (relative humidity 65 per cent) and were observed several times daily throughout the course of the experiments for possible signs and symptoms of encephalitis. When tissues were obtained for virus assay, extreme care was taken to rid specimens of as much blood as possible so that resulting virus titers would reflect virus multiplication in a specific tissue and not virus present in blood circulating through that tissue. These experiments, which will be published in detail elsewhere (Sulkin, Allen and Sims, unpublished data), indicate that the Mexican free-tailed bat is only slightly susceptible to the high mouse passage Nakayama strain of Japanese B encephalitis virus, but is significantly more susceptible to a mosquito isolate (OCT-541) which had been through two hamster kidney tissue culture passages and one passage in little brown Myotis. In the latter case, the infection was widely disseminated in animals sacrificed over a period of 3 weeks, with virus concentrations reaching 3 logs or more in blood, brown fat, and kidney. In one instance virus was demonstrated in the brown fat and not in the

VIRAL INFECTION IN BATS

blood of an animal sacrificed 21 days after virus inoculation. The passage history of the virus strain seems to determine its degree of infectivity for the Mexican free-tailed bat, where the recently isolated strain is much more infective than the laboratory-adapted strain. Similar results were obtained with two strains of St. Louis encephalitis virus. This bat species is quite resistant to infection with the high mouse passage Hubbard strain, but is quite susceptible to infection with a strain recovered from a flicker bird and in its seventh mouse brain passage. Virus was first demonstrated in blood one to two days after virus inoculation, increased to levels of 4.0 log units by the seventh day, and was still detectable in animals sacrificed about one month post-inoculation. In addition, virus was demonstrated with much greater frequency in brown fat than in brain or kidney. In a few instances virus was demonstrated in tissue preparations from animals that were not viremic at the time of sacrifice.

The little brown bat is highly susceptible to the OCT-541 strain of Japanese B encephalitis virus. Virus was widely distributed in the various tissues assayed during an observation period of about one month; virus was demonstrated in the blood and in brown fat with about equal frequency, and in several instances virus was demonstrated in the brown adipose tissue and not in blood or other tissues tested. This bat species is only slightly susceptible to infection with the flicker bird strain of St. Louis encephalitis virus. The big brown bat (*Eptesicus f. fuscus*) is highly susceptible to infection with the OCT-541 strain of Japanese B encephalitis virus. All animals developed widespread infection during the first 12 days following virus inoculation, titers in the blood and brown fat reaching more than 3.0 log units in many instances. Subsequent to the twelfth day, the infection seemed gradually to subside; only 2 of 10 animals tested had a detectable viremia by the twenty-fifth day. The susceptibility of this bat species to the other virus strains has not yet been determined.

SULKIN AND ALLEN

INFLUENCE OF TEMPERATURE ON
ANTIBODY PRODUCTION IN BATS

In planning studies on the influence of hibernation on the progress of these experimental viral infections in bats, it seemed desirable to determine the pattern of antibody production in these animals following peripheral inoculation of various arthropod-borne viruses. As pointed out earlier, the unique thermoregulatory mechanism of bats sets them apart from other animals and suggests this animal as an ideal experimental host whose temperature and metabolism may be altered at will, permitting the influence of temperature on infection and antibody production to be studied over extended periods. The use of bats also permits the achievement of hypothermia without the use of drugs or surgery which might have an independent effect on the physiochemical balance of the host.

A bacterial antigen was selected for a pilot experiment on the effect of temperature on antibody production because of the ease with which one can demonstrate agglutinating antibodies. The typhoid-H antigen-antibody system was chosen as representing a relatively simple immunization procedure and antibody assay technique requiring very small serum samples, and the big brown bat was used because it can withstand repeated bleeding by cardiac puncture. One group of bats was maintained at room temperature (26°C) and another in simulated hibernation (10°C). Rectal temperatures were taken periodically. All bats received 3 intraperitoneal doses of 0.25 ml each of an 8-hour formalized broth culture of Salmonella typhosa on days 0, 15 and 24 of the experiment. Several animals were bled periodically and all were bled on the 35th day following the initial immunizing dose. The antibody level of each serum sample was determined by means of the tube agglutination test. The results indicate that the ability of the big brown bat to produce antibody in response to injections of a bacterial antigen is dependent upon its body temperature, which parallels that of the environment (Fletcher et al., 1962). After 35 days and 3 doses of antigen, over 70 per cent of the animals had agglutinin titers ranging from 1:8 to 1:672. In striking contrast, the bats held at 10°C failed to produce any detectable antibody during this period. Some animals which had been

VIRAL INFECTION IN BATS

maintained at room temperature were transferred to the cold room on the 42nd day, and blood specimens were obtained one month later. Persistence of antibody was observed in 5 bats, but in each instance agglutinin titer dropped. Bats kept in simulated hibernation for 42 days were transferred to room temperature and one month later (or 72 days after initiation of the experiment) blood samples were obtained. Although no additional antigen was administered upon transfer to room temperature, 6 of 10 animals uniformly negative after 30 days in the cold possessed agglutinating antibody one month after transfer to the warm room. Presumably, antigen persisted during hibernation.

Several studies have been made to determine the immune response of big brown bats experimentally infected with Japanese B encephalitis virus and held at room temperature or in simulated hibernation for extended periods. Results indicate an extremely close association between the cessation of viremia and the appearance of detectable neutralizing antibody. Even virus may be detected in low titer in blood samples containing neutralizing antibody. Also, preliminary studies indicate that bats experimentally infected with this virus do not regularly produce antibodies detectable by the complement fixation (CF) and hemagglutination-inhibition (HI) techniques currently in use. Experiments designed to study the antibody response to the hibernating bat and the fate of any neutralizing antibody present in the animals entering hibernation indicate that antibody is produced by bats kept at room temperature, while none is detectable in those held in the cold. As in the case with the bacterial antigen, there is evidence that transfer to room temperature stimulates antibody syntheses. In general, it has been found that bats which had equivocal titers (1.0 to 1.7 LNT)² at 17 to 21 days were usually strongly positive about 2 months post-inoculation. Although viremia develops in all experimentally infected bats, some fail to develop even equivocal titers. As was the case in experiments with the bacterial antigen, none of the bats produced neutralizing antibody in response to Japanese B encephalitis virus during 43 days at 10° C. Similar results were obtained with some bats which were inoculated with rabies virus and bled after 43 days in the cold (Sulkin et al., 1960).

² Log neutralization index (LNT). An LNT of < 1.0 was considered negative, 1.0 to 1.7 equivocal, and 1.7 or above positive.

SULKIN AND ALLEN

INFLUENCE OF HIBERNATION ON INFECTION AND ANTIBODY
RESPONSE OF BIG BROWN BATS INOCULATED WITH
JAPANESE B ENCEPHALITIS VIRUS

Having established the susceptibility of the big brown bat (*Eptesicus f. fuscus*) to experimental infection with Japanese B encephalitis virus and determined the duration of the viremic phase, the degree of involvement of brown adipose tissue, brain, and kidney, and learned something of the antibody response of infected animals held at room temperature, the influence of low temperature on this infectious process could then be studied. In a study on Japanese B encephalitis infection in bats during simulated hibernation, LaMotte (1958) reported that viremia is suppressed in animals placed at 10° C immediately after inoculation; upon transfer to room temperature after as long as 3 months in the cold, demonstrable levels of virus appeared in the blood within 2 to 5 days.

In designing our studies, we have attempted to reproduce in the laboratory situations as they might occur in nature. Whenever possible, experiments are planned so that the periods in which animals are held at low temperature in the laboratory correspond to the season of natural hibernation in an effort to simulate hibernation rather than merely attain a state of hypothermia (Menaker, 1962). Groups of bats are placed in cold rooms at varying times following virus inoculation to represent (1) animals entering hibernation immediately after becoming infected and before infection develops to a demonstrable level; (2) animals entering hibernation at the peak of the infectious cycle when virus is present in blood and other tissues; and (3) animals entering hibernation while in the immune phase of the infection when virus is no longer regularly demonstrable in blood or other tissues and significant levels of neutralizing antibody are present. Although these studies are still in progress, we have analyzed sufficient data to enable us to construct a schematic diagram which we believe to be representative of experimental infection with this virus in big brown bats under the various environmental conditions described (Fig. 3).

The uppermost section in Figure 3 depicts infection and antibody

VIRAL INFECTION IN BATS

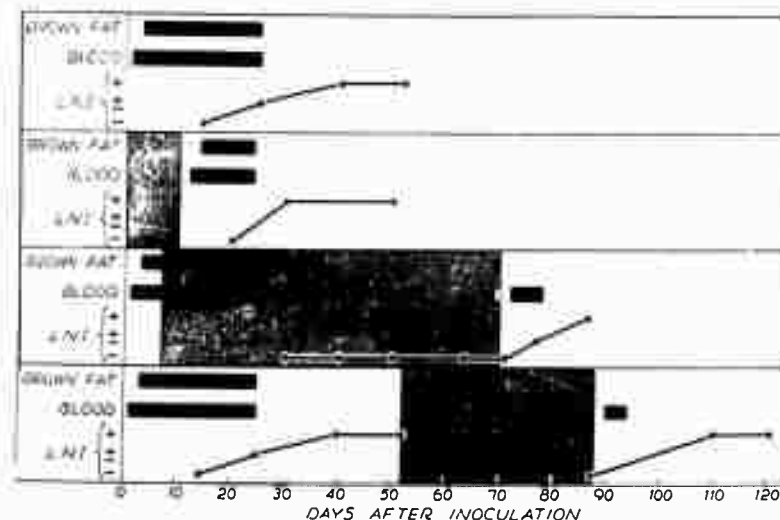


Figure 3. Schematic representation indicating suppressive effect of simulated hibernation on progress of infection and immunologic response of bats (*Eptesicus f. fuscus*) following intramuscular inoculation of Japanese B encephalitis virus (150 weanling mouse i. c. LD₅₀). Shaded areas refer to periods in simulated hibernation (10° C). Solid bars indicate virus demonstrated by intracerebral inoculation of weanling mice. Clear bar indicates no virus recovered. Log neutralization index (LNI) indicated by curves.

response in animals held at room temperature from time of inoculation. Some animals become viremic as early as 24 to 48 hours after receiving 150 LD₅₀ doses of virus, whereas virus is seldom isolated from brown adipose tissue before the 3rd or 4th day post-inoculation. We indicate demonstration of virus in blood and brown fat over a period of approximately 25 days. It is difficult to define precisely the duration of active infection, since there appears to be much individual variation. We do know that virus can be demonstrated in brown fat and/or blood of virtually all animals tested 7 to 12 days after receiving virus and that subsequently, the number of infected animals and the concentrations of virus in their tissues gradually diminish. This decrease in viral proliferation coincides with the time when neutralizing antibodies first become detectable, and it is at this point that we have demonstrated on occasion low concentrations of virus in the blood of animals with positive neutralization indices. A significant number of animals have positive LNI 30 to 50 days post-inoculation, although some infected animals never develop antibody detectable by the methods used. Studies on the

SULKIN AND ALLEN

persistence of antibody and the occurrence of cyclic episodes of infection in animals held at room temperature are incomplete at this time.

In the remaining sections of the figure, the shaded areas indicate the periods following injection of virus when bats are maintained at low temperature (10°C) and the influence of these periods of simulated hibernation on the progress of infection has been plotted. When animals are placed at 10°C immediately after inoculation, viral multiplication is suppressed. Occasionally, one can demonstrate a trace of virus in blood or brown fat, but it is difficult to determine by assay of specimens in mice the fate of the virus inoculum during this period in the cold. When the animals are transferred to room temperature, virus reaches demonstrable levels in blood and brown fat 2 to 3 days later. It appears that the infection which occurs following a period of suppression in the cold is of shorter duration than in room temperature groups, and antibody reaches significant levels more rapidly. On the other hand, bats placed at 10°C one week after virus inoculation, while at the peak of the infection cycle, seem to suffer prolonged infection. Virus can be isolated regularly from brown fat and blood for at least 30 days after the animals are placed in the cold. Although subsequent data are incomplete, we have included an instance in which virus was isolated from the brown adipose tissue of a bat more than 2 months after the animal was placed in simulated hibernation. The blood of this animal did not contain demonstrable virus, but companion animals circulated virus 3 days after transfer to room temperature. None of the infected bats developed antibody while in the cold, but plasma samples with positive LNI were obtained from bats in this group approximately 2 weeks after transfer to room temperature. In still another series of experiments mapped in the bottom section of Figure 3 we are attempting to determine if an animal entering hibernation in an immune state could, upon arousal, circulate virus again spontaneously. Data along these lines accumulate slowly, since infected bats must be held at room temperature 30 to 50 days for antibody development, then withstand an additional month or more at 10°C and survive transfer back to room temperature before the end results can be obtained. At the present time we have some data indicating that at least some bats with positive LNI, when placed in the cold, lose their antibody during a period of a month or more in hibernation

VIRAL INFECTION IN BATS

and upon transfer to room temperature 3 months after the initiation of the original infection may experience a second cycle of infection and antibody response.

DISCUSSION AND SUMMARY

Our primary interest in studying the influence of low temperature on experimental viral infections in bats has been to accumulate information which would help in understanding how periods of winter hibernation could affect natural virus infections in these animals. As might be expected, the depressed metabolic state and the low body temperature of bats held at 5° C or 10° C are not conducive to active viral multiplication. Of far greater interest is the observation that bats in simulated hibernation are able to sustain viral infections during this period of inactivity. Although virus does not appear to replicate at a readily measurable rate during this period, viability is maintained. Upon transfer to a warmer environment simulating spring arousal, virus lying dormant in brown adipose and/or other tissues begins to multiply and active infection, demonstrable by conventional assay methods, ensues. This sustenance of viral particles in the hibernating bat seems more remarkable when accomplished in animals placed at low temperature immediately after virus inoculation. In our studies with rabies virus, animals were inoculated intramuscularly and placed immediately at 5° C or 10° C, allowing no time for virus to attach to or penetrate the cells of the animals at room temperature or the higher temperatures believed to be optimum for this animal virus; yet some bats transferred to a warm environment even a month or more later were subsequently shown to develop rabies infection. During the periods inoculated animals were held at low temperature, only an occasional animal could be proved infected upon sacrifice, and in all cases rabies virus was demonstrated in low titer, indicating that virus was not multiplying very rapidly in these bats. Evidence was obtained, however, in an experiment with a bat rabies virus strain in little brown *Myotis*, that some type of viral activity occurred during the period the inoculated animals were held at 5° C which resulted in what appears to

SULKIN AND ALLEN

be a modification of the virus strain, Sadler and Enright (1959) have recorded the suppressive effect of low temperature on rabies infection in bats and have correlated it with the lowered metabolic rate of the animals. These investigators found no evidence of rabies virus multiplication in animals placed at low temperature immediately after virus inoculation, but if infection were allowed to develop for 6 days at 22° C prior to transfer of animals to 4° C, some evidence was obtained of virus multiplication in the brains of animals which died after 30 to 90 days in the cold.

The experimental data recorded to date on the influence of low temperature on rabies virus infection in bats suggest that winter hibernation may have a profound effect on natural rabies infections in these animals. There is no doubt that the period of winter dormancy could exert a sparing effect perhaps sufficient to maintain a constant state of latency in a large population. In addition, there is the suggestion that rabies virus particles which overwinter in the tissues of the bat and are subject to either complete dormancy or a shift to a much decreased rate of multiplication may be altered by this period at below optimum temperature.

Because the big brown bat is highly susceptible to Japanese B encephalitis virus and suffers an infection which can be characterized as to incubation time, duration of the initial infectious phase, and antibody response, the format of our low temperature studies with this virus-host system is more inclusive than has been possible in work with rabies virus. Assuming that in a given population animals would vary as to the stage of their natural arbovirus infection, we are studying the effects of simulated hibernation on groups of animals which are pre-viremic, viremic or post-viremic, and immune. The results of these studies allow us to speculate along the following lines: (1) bats which enter hibernation soon after receiving an infective dose of Japanese B encephalitis virus, but before infection has developed to demonstrable levels, would be capable of sustaining infection through months of winter hibernation and upon arousal in the spring provide infective blood for feeding vectors; (2) bats which enter hibernation with high titers of Japanese B encephalitis virus in blood and tissues apparently suffer a prolonged infection, possibly due to the suppression of antibody production in the cold; (These animals could provide infective blood for at least a month for mos-

VIRAL INFECTION IN BATS

quitoes present at the hibernating site. In this regard, it is of interest that LaMotte (1958) has shown that mosquitoes will feed on bats at 10°C. Thus the viremic, hibernating bat could provide a mid-winter feeding of infected blood for mosquitoes, making possible a two-step winter transmission chain as suggested by Bellamy, Reeves and Scrivani (1958) for western equine encephalitis virus in birds and mosquitoes. In addition, the bat which enters hibernation in a viremic state, although his blood titer gradually decreases during the winter, would be capable of circulating virus again upon arousal in the spring) and (3) bats which enter hibernation at a time when they possess neutralizing antibodies against Japanese B encephalitis virus may show negative neutralization indices by the termination of their winter sleep and the warmer environmental temperature, together with the physiological alterations which occur at time of arousal and emergence, could serve to activate latent Japanese encephalitis virus infection and produce viremic hosts without the necessity of re-infection.

We feel that these studies demonstrating experimentally the various conditions under which the hibernating bat can serve to overwinter an arbovirus add strong supportive evidence to the growing concept of the role of hibernating animals in the maintenance of these agents in nature during periods when vectors are not active. Another hibernating mammal, the hedgehog, has been shown experimentally to circulate Russian spring-summer encephalitis virus during periods in the cold (van Tongeren, 1958) and experimental studies with western equine encephalitis virus in snakes (Thomas and Eklund, 1960) and Japanese B encephalitis in frogs (Chang, 1958) suggest that these poikilothermic animals may be capable of overwintering these viruses.

In addition to the broad epidemiological aspects of the studies presented here, the compiled data also provide information of a more fundamental nature relative to the influence of temperature on the virus-cell-host relationship per se. There is no doubt that the multiplication rate of both rabies and Japanese B encephalitis viruses is suppressed in the bat by maintaining the animal at low temperature. The manner in which infection with both these agents is sustained for long periods of time in the dormant animal, however, suggests that some type of viral activity occurs in this host at low

SULKIN AND ALLEN

temperature. The apparent alteration in the bat rabies virus strain which occurred as a result of a period of "incubation" in bats at 5° C further supports this concept. There is also indication that the Japanese B encephalitis virus may be altered by passage through bats at 10° C. The virus demonstrable in the blood and other tissues of these animals upon transfer to 24° C often produces a disease in mice characterized by increased incubation time, bizarre paralytic symptoms and frequently, recovery. In experiments now in progress we are studying strains of viruses following single and multiple passages through bats held at low temperature. These *in vivo* studies are being paralleled with *in vitro* experiments using monolayer and explant cultures of bat brown fat, kidney and embryonic tissue, as well as preparations of tissues from warm-blooded animals in an effort to produce virus strains altered by passage at low temperature in different host systems. A study of virus strains altered by the pressures of low temperature exerted in the intact bat, cultures of bat tissues, and cultures of tissues from warm-blooded animals should provide definitive information concerning the mechanisms of temperature-induced variations in animal viruses.

LITERATURE CITED

1. Bell, J. F., and G. J. Moore. 1960. Rabies virus isolated from brown fat of naturally infected bats. *Proc. Soc. Exp. Biol. Med.* 103: 140-142.
2. Bellamy, R. E., W. C. Reeves, and R. P. Scrivani. 1958. Relationships of mosquito vectors to winter survival of encephalitis viruses. II. Under experimental conditions. *Am. J. Hyg.* 67: 90-100.
3. Chang, I-C. 1958. Studies on Japanese B encephalitis in cold-blooded animals. *Pediatrics* 4: 27-49.
4. Corrigan, E., L. LaMotte, Jr., and D. G. Smith. 1956. Susceptibility of bats to certain encephalitis viruses. *Fed. Proc.* 15:584.

VIRAL INFECTION IN BATS

5. Enright, J. B. 1956. Bats and their relation to rabies. *Ann. Rev. Microbiol.* 10: 369-392.
6. Fawcett, D. W. 1952. A comparison of the histological organization and cytochemical reactions of brown and white adipose tissues. *J. Morphol.* 90: 363-405.
7. Fletcher, Mary Ann, Ruth Sims, Rae Allen, and S. Edward Sulkin. 1962. Influence of temperature on antibody production in the bat. *Texas Rep. Biol. Med.* 20: 142.
8. Griffin, Donald R. 1958. *Listening in the Dark*. Yale Univ. Press.
9. Grodums, Irene, and George Dempster. 1959. The age factor in experimental Coxsackie B-3 infection. *Canad. J. Microbiol.* 5: 595-604.
10. Hock, R. J. 1951. The metabolic rates and body temperatures of bats. *Biol. Bull.* 101: 289-299.
11. Hock, R. J. 1958. Hibernation. p. 61-133. In: *Cold injury*. Trans. 5th Conf. Josiah Macy Found.
12. Hoggan, M. D., and B. Roizman. 1959. The effect of the temperature of incubation on the formation and release of herpes simplex virus in infected FL cells. *Virology* 8: 508.
13. Ito, T., and S. Salto. 1952. Susceptibility of bats to Japanese B encephalitis virus. *Jap. J. Bact.* 7: 617-622.
14. Johansson, B. 1959. Brown fat: a review. *Metabolism* 8: 221-240.
15. Johansson, Bengt. 1960. Brown fat and its possible significance for hibernation. p. 233-248. In *mammalian hibernation*. Lyman and Dawe, eds. Cambridge, Mass.
16. Kayser, C. 1961. *The physiology of natural hibernation*. Pergamon Press, New York. v 8.

SULKIN AND ALLEN

17. LaMotte, L. C., Jr. 1958. Japanese Bencephalitis in bats during simulated hibernation. *Am. J. Hyg.* 67: 101-108.
18. Lwoff, A. 1959. Factors influencing the evolution of viral diseases at the cellular level and in the organism. *Bact. Rev.* 23: 109-124.
19. Menaker, Michael. 1962. Hibernation-Hypothermia: An annual cycle of response to low temperature in the bat Myotis lucifugus. *J. Cell. Comp. Physiol.* 59: 163-173.
20. Morrison, P. 1959. Body temperatures in some Australian mammals. I. Chiroptera. *Biol. Bull.* 116: 484-497.
21. Pappenheimer, A. M., J. B. Daniels, F. S. Cheever, and T. H. Weller. 1950. Lesions caused in suckling mice by certain viruses isolated from cases of so-called non-paralytic poliomyelitis and of pleurodynia. *J. Exp. Med.* 92: 169-190.
22. Rasmussen, A. T. 1923. The so-called hibernating gland. *J. Morphol.* 38: 147-205.
23. Remillard, G. L. 1958. Histochemical and microchemical observations on the lipids of the interscapular brown fat of the female vesperilionid bat Myotis lucifugus. *Ann. N. Y. Acad. Sci.* 72: 1-68.
24. Sadler, W. W., and J. B. Enright. 1959. Effect of metabolic level of the host upon the pathogenesis of rabies in the bat. *J. Infect. Dis.* 105: 267-273.
25. Schwartzman, G. 1952. Poliomyelitis infection in cortison-treated hamsters induced by the intraperitoneal route. *Proc. Soc. Exp. Biol. Med.* 79: 573-576.
26. Sims, Ruth, Rae Allen, and S. E. Sulkin. 1963. Studies on the pathogenesis of rabies in insectivorous bats. III. Influence of the gravid state. Unpublished data.

VIRAL INFECTION IN BATS

27. Sulkin, S. E. 1945. The effect of environmental temperature on experimental influenza in mice. *J. Immunol.* 51: 291-300.
28. Sulkin, S. E., and M. J. Greve. 1954. Human rabies caused by bat bits. *Texas State J. Med.* 50: 620-621.
29. Sulkin, S. E., P. H. Krutzsch, R. Allen, and C. Wallis. 1959. Studies on the pathogenesis of rabies in insectivorous bats. I. Role of brown adipose tissue. *J. Exp. Med.* 110: 369-388.
30. Sulkin, S. E., Rae Allen, Ruth A. Sims, P. H. Krutzsch, and C. Kim. 1960. Studies on the pathogenesis of rabies in insectivorous bats. II. Influence of environmental temperature. *J. Exp. Med.* 112: 595-617.
31. Sulkin, S. E., R. Allen, R. Sims. 1960. Lipotropism in pathogenesis of encephalitis viruses in insectivorous bats. *Virology* 11: 302-306.
32. Sulkin, S. E. 1962. The bat as a reservoir of viruses in nature. v.4. In *Progress in Medical Virology*. Karger/Basel, New York.
33. Sulkin, S. E., Rae Allen, and Ruth Sims. Studies of arthropod-borne virus infections in Chiroptera. I. Susceptibility of insectivorous species to experimental infection with Japanese B and St. Louis encephalitis viruses. Unpublished data.
34. Thomas, Leo A., and Carl M. Ecklund. 1960. Overwintering of western equine encephalomyelitis virus in experimentally infected garter snakes and transmission to mosquitoes. *Proc. Soc. Exp. Biol. Med.* 105: 52-55.
35. Tongeren, H. A. E. van. 1958. Sixth International Congress on Tropical Medicine and Malaria. (Abstr.) Lisbon, 5-13 September p. 166.
36. Walker, D. L., and W. D. Boring. 1958. Factors influencing host-virus interactions. III. Further studies on the alteration of Coxsackie virus infection in adult mice by environmental temperature. *J. Immunol.* 80: 39-44.

SULKIN AND ALLEN

DISCUSSION

CAMPBELL: Did you do electrophoretic patterns of the hibernating bats?

SULKIN: We are doing them now, Dr. Campbell, and we are finding things that I haven't yet had time to sit down and figure out, but we are seeing some very strange things in these electrophoretic patterns. I really don't know what to make of it.

ANDREWES: I understand that viremia is absent in hibernating snakes and then comes back again, as in your bats. When they come out of hibernation, is there any evidence in your bats as to whether this is a function of temperature or not; or hasn't that been done?

SULKIN: I don't think we have enough data. Perhaps Dr. Marcus knows. I don't think so.

SCHMIDT: Many times you used the term "simulated hibernation". Would you care to define it?

SULKIN: I suppose I could delete the word "simulated", but I think that when you take a bat away from his natural environment and then try to simulate these circumstances in the laboratory, there is a difference. I think that when we net the bats in the fall at the time when they ordinarily would be going into hibernation, we are dealing with true hibernation. This is not the case when such experiments are carried out with bats netted in the summer. Such animals, when placed at low temperatures, are hypothermic.¹

WALKER: You don't see any difference in your experiments, though, in these two seasons?

¹ Menaker, J. 1962. *Cell and Comp. Physiol.* 59: 163-173.

VIRAL INFECTION IN BATS

SULKIN: Well, there are many differences in the same bat species, Eptesicus fuscus. You can do an experiment of this sort. You can collect bats in the fall of the year and use thermocouples and record body temperature of these bats. Now, if you take a bat just prior to the time he is going into hibernation, and then place him at about 8° C to 10° C in the laboratory, then record temperatures over a period of time, rectal temperature will march along at a very steady state. If you stimulate this bat with a pair of forceps, the temperature goes up, and goes up very promptly. If you take the same species, Eptesicus fuscus, the big brown bat, and you do the same experiment, but do it in July instead using the same probe and the same amount of insertion into the rectum and so on, everything identically the same, then you can stimulate this bat and nothing happens. We have done this with many bats. So this is one significant difference between the true hibernating bat and the simulated hibernating bat.

TRAPANI: Don't they wake up during the winter time for feeding?

SULKIN: No, they stay in the cage.

TRAPANI: In their natural habitat?

SULKIN: In their natural habitat they don't feed; they store food in their brown fat, presumably, and this is enough to keep them going. One can do this with hamsters, too, but it's a trick.

BERRY: In Texas, doesn't the temperature get high enough to bring them out of the hibernating state?

SULKIN: Most of the bats in Texas are not true hibernating species, but migratory species. During mid-winter they migrate south to an area where the temperature is optimum.

BERRY: But bats are in the caves in Texas in the winter time.

SULKIN: Yes, many Mexican free-tailed bats can be found in

SULKIN AND ALLEN

certain of the caves during the winter time.

BERRY: But they can't be hibernating all the time, can they?

SULKIN: Most of the bats in Texas, and there are millions and millions of Mexican free-tails there, are not of a true hibernating species. They go out for night time feeding. Consistent with that is the fact that they have very little brown fat in the interscapular region, so it's very difficult to do experiments with this particular species.

BERRY: Just out of curiosity, how do you feed them?

SULKIN: The big brown bats (Eptesicus f. fuscus) are hand-fed meal worms the first week we get them into the laboratory. Most of them learn to feed in about 2 to 3 days. Then we put them on a concoction that we have prepared which consists of cottage cheese, banana, and meal worms, to which is added liver extract with iron and multivitamins.

BERRY: They won't breed?

SULKIN: No. They don't breed under laboratory conditions, but many of the gravid bats have delivered their young in the laboratory.

SCHMIDT: Concerning true hibernation or simulated hibernation in the same species, I believe you indicated that if you induce this simulated hibernation during the summer months, this animal is then under stress and not truly hibernating. I am wondering if he is burning excessive amounts of body tissue of some sort to accommodate himself. Have you made any measurements of weight loss comparing these two periods of time?

SULKIN: No, we haven't done any good experiments along this line. We are so convinced that this is a different host when induced to hibernate during the summertime that we do these experiments on a yearly basis in the late fall.

VIRAL INFECTION IN BATS

CAMPBELL: Have you ever injected the brown fat?

SULKIN: Yes, into a non-hibernating animal. The experiments don't work too well. Two sets of experiments have been reported previously, and conflicting results were obtained.^{2,3}

BLAIR: Bigelow has done this in Toronto.

SULKIN: These are extracts, and they are not very pure.

BLAIR: He transplanted the brown fat and the results were entirely negative.

SULKIN: Nobody has as yet been able to define the active component in brown fat tissue that is likely to be related to hibernation. And I think that there is a growing interest in this tissue as the true mechanism of hibernation.

TRAPANI: One thing we tried which seemed to indicate some activity of brown fat was this: We made a saline extract out of brown fat obtained from the Guinea pigs exposed to 2° C for about 10 weeks. Ordinarily, you put Guinea pigs into -15° C and they don't do very well; they die quickly. However, when saline extract of brown fat was injected into animals kept at -15° C, they survived another day or so. We never tried it again because of the lack of time.

SULKIN: I think that if you tried it again, it might not work. When saline extracts of brown fat were used by previous investigators, they yielded inconsistent results.

ANDREWES: We have been hearing quite a lot in the last two days on the subject of cold and other stressing factors. There is one form of stress that has never been mentioned. There were some experiments done a good many years ago on pneumococcal infections in partly-immunized mice when it was shown

2 Zirm, 1956. *Zschr. f. Naturforsch.*, 11: 530-535.

3 Kross, 1933. *Zschr. f. d. ges. Neurol. Psych.* 146: 208-218.

SULKIN AND ALLEN

that these infections could be activated by the stress of alcohol. This is a subject which needs further investigation.

MICROBIOLOGICAL ASPECTS OF HIBERNATION IN GROUND SQUIRRELS

J. Schmidt

Department of Bacteriology
University of New Hampshire
Durham, New Hampshire

ABSTRACT

Studies were designed to determine the effect of reduced temperatures on the normal bacterial and viral flora of the experimental animals and on the fate of other microorganisms artificially introduced. Determinations were made of the number of coliform bacilli, fecal streptococci, and psychrophilic organisms present in the fecal material before and immediately after hibernation. In addition, the total viable aerobic cell count was determined. All counts were based on the number of organisms per gram (dry weight) of fecal material. The data indicate a gradual 3 log increase in the number of psychrophiles and a simultaneous decrease of similar magnitude in the number of coliform bacilli during periods of hibernation. No change which could be associated with hibernation was noted in either the total cell count or the number of fecal streptococci. The animals were shown to be free of viruses capable of inducing a cytopathogenic effect in monolayers of either HeLa, human amnion, or monkey kidney cells. Fecal samples from each of 32 animals were repeatedly tested over a period of four months. Bacterial viruses (*Escherichia coli* phage) have been demonstrated in only one of the 40 ground squirrels examined. Several strains of *E. coli* isolated from the animals were used as the host cell in the test systems. The effect of hibernation on the presence of artificially introduced ECHO-6 and Coxsackie B-3 viruses and *E. coli* phage in the intestinal tract of ground squirrels is being evaluated.

Hibernating mammals provide an excellent means for studying the effect of cold on the biology of experimental infection from the standpoint of both the host and the parasite. These animals represent a unique situation in which a normal system is available in the same species, at widely separated temperatures. For instance, in the active state a ground squirrel has a normal body temperature of approximately 37° C, whereas in hibernation its normal temperature may approach 0° C.

The studies reported here deal with the effect of hibernation on

SCHMIDT

certain components of the normal bacterial and viral flora and on the retention of specific viral agents in the intestinal tract of ground squirrels.

Since hibernation is such an important feature of these investigations, a few words about some of its salient characteristics are in order. First of all, it is important to recognize that hibernation is a natural phenomenon for certain animals and that it is quite different from experimental hypothermia. Hibernation is a physiologically controlled and regulated process, while experimental hypothermia is a result of weakened or overcome normal mechanisms of temperature regulation. Chilling occurs in enforced hypothermia in spite of a concerted effort on the part of the animal to maintain its normal temperature. On the contrary, hibernation is a passive, yet deliberate process in which declines in metabolic rate, respiratory rate, and heart rate precede the drop in body temperature.

Within a limited range, the body temperature of the hibernating ground squirrel parallels that of the environment, usually remaining 0.5°C to 3°C above the ambient temperature (Johnson, 1931). Mayer (1960) reported an average rectal temperature of 4.2°C for ground squirrels hibernating in an environmental temperature of 1.6°C . The heart rate and respiratory rate are slowed to about 3 per min, and the metabolic rate is between $1/30$ and $1/100$ of that found in the active "resting" animal. Vascular changes include a decrease in blood pressure as the animal enters hibernation followed by vasoconstriction once the torpid state has been achieved (Lyman and O'Brien, 1960). There appears to be a pronounced leucopenia during hibernation (Svihla, 1953) which has not been explained. Although the rate is very slow, Brock (1960) reports that there is actually some manufacture of red blood cells during deep hibernation.

There are indications that some of the endocrines play a role in hibernation, although the specific data are conflicting. Most workers agree that an involution of the endocrines takes place before the animal enters hibernation. Indeed, Kayser (1955) insists that hibernation will not take place without this involution.

Although hibernation is part of a yearly cycle of events for ground squirrels, it is not clearly understood what specific factors are im-

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

portant in determining when they will enter or emerge from the hibernating state. It would appear that the animal receives few, if any, clues from its environment, such as photoperiodicity or temperature (Pengelley and Fischer, 1957).

Ground squirrels and other mammalian hibernators undergo natural awakenings from time to time during the hibernating period (Lyman and Chatfield, 1955). Kayser (1960) reported that the average period of continuous hibernation in ground squirrels is about 21 days. In our work we have been somewhat less successful. The cause of the periodic arousals in undisturbed animals has yet to be explained.

For a more complete survey of the physiology of mammalian hibernation, the reader is referred to the recent publication edited by Lyman and Dawe (1960).

MATERIALS AND METHODS

Arctic ground squirrels (*Spermophilus undulatus*), adult animals of both sexes, were captured in the Paxson Lake area of central Alaska during the month of August. They were housed individually in metal cages equipped with removable wire mesh floors and catch pans. Their diet consisted of apples, carrots, and Labena pellets (Ralston Purina Co., St. Louis, Mo.). They were observed in the colony for 2 months before the studies were initiated. Hibernation was induced by placing the animals in a cold room at an ambient temperature of $3^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for the bacterial work and $5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for the viral studies. These temperatures were maintained throughout each of the respective investigations. The animals were observed daily but not disturbed unnecessarily, because various stimuli may trigger an arousal from the hibernating state.

The primary explants of monkey kidney cells were obtained from Shamrock Farms (Middletown, New York) and maintained on Eagles basal medium with Hank's balanced salt solution (BSS). HeLa monolayers were prepared from the S-3 clone, kindly supplied by

SCHMIDT

Dr. John L. Riggs of the University of Michigan, using 90 per cent Eagles BM and 10 per cent calf serum as the growth medium. For maintenance, the calf serum was reduced to 5 per cent. The same growth and maintenance media were used for the cultures of the RA strain of human amnion cells.

The specific viral agents used were the D'Amori strain of ECHO-6 virus and the Nancy m₅K₃ strain of Coxsackie B-3.

Bacterial Studies

Investigations were made of the normal intestinal flora of 15 adult ground squirrels both in the active state and following periods of deep hibernation. Fresh fecal droppings were collected on filter paper, and those contaminated with voided urine were excluded. Specimens were kept at 4° C until processing, which took place within 2 hours after defecation. The droppings from an individual animal were weighed and combined with an equal weight of sterile distilled water. From this a uniform suspension was made with the aid of a sterile glass rod. Part of the material was used for the determination of the per cent moisture and another portion for the preparation of cultures. Triplicate cultures were inoculated from serial 10-fold dilutions (10^{-3} to 10^{-8}) of the specimens, and the results obtained were calculated as the number of organisms per gram dry weight of fecal material. Quantitative determinations were made of the coliform bacilli, fecal streptococci, and psychophilic organisms, in addition to a total viable aerobic cell count. The coliform bacilli were cultured on (Difco) eosin methylene blue agar at 37° C. The plates were seeded with 0.1 ml of the appropriate dilution which was then spread evenly over the surface of the agar with a bent glass rod. The inoculum was dispensed with a sterile 0.1 ml pipette. Using this technique, well-isolated colonies were consistently obtained. All gram negative rods yielding lactose-positive colonies within 48 hours were arbitrarily considered to be coliform bacilli.

For the enumeration of the psychophiles, (Difco) tryptone glucose extract (TGE) agar was employed. Pour plates were prepared using an inoculum of 1 ml and the incubation was carried out at 2° C to 3° C for 14 days. Organisms producing a visible colony within 14

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

days were termed psychrophiles. In addition, pour plates were made in TGE agar as above, except that these cultures were incubated at 37° C for 48 hours. The results from these latter cultures were referred to as the total count at 37° C. The number of fecal streptococci was determined using (Difco) azide dextrose agar with an inoculum of 1 ml and 37° C incubation temperature. All plate counts were made using a Quebec colony counter and only those plates having between 30 and 300 colonies were included.

Bacterial Virus Study

In order to gain some information concerning the normal occurrence of bacterial viruses in the animals, 44 adult ground squirrels were examined repeatedly (154 individual samples) for the presence of bacteriophage in their intestinal contents. The bacterial host cells used were strains of Escherichia coli previously isolated from the animals in the group.

The method used for the detection of phage was as follows. Fresh fecal material was collected in a sterile tarred 50 ml centrifuge tube and the weight of the sample determined. A one to 10 dilution (weight in volume) was prepared in sterile nutrient broth and a uniform suspension made with the aid of a sterile glass rod. Following centrifugation at 17,000 X g for 30 min at 5° C the clear supernatant fluid was withdrawn and tested for the presence of virus by two methods. In the first, 1 ml of the fluid and 1 ml of a 3 hr broth culture of the strain of E. coli being tested was added to 5 ml of trypticase soy broth. This suspension was incubated overnight at 37° C and the supernatant fluid therefrom was assayed for the presence of virus according to the following procedure. Serial 10-fold dilutions (10^{-1} to 10^{-6}) of the fluid were prepared in sterile nutrient broth and 0.1 ml added to a tube containing 2 ml of melted (43° C) trypticase soy agar (0.7 per cent) and 0.1 ml of an 18 hour broth culture of the test strain of E. coli. This was carefully mixed and poured over the surface of a layer of nutrient agar in a petri plate. These cultures were incubated at 37° C and examined for the presence of virus as indicated by the formation of plaques. In the second method the preliminary overnight incubation was eliminated, and the original supernatant fluid was tested for bacterial virus by the plaque method.

SCHMIDT

These rather serious attempts to isolate phage disclosed that only one ground squirrel out of the 44 tested was excreting a bacterial virus reactive against any of the host strains used. This virus (E. coli phage 42) would effectively lyse our strain 14 of E. coli. Therefore, we had available a working system which had been isolated from the colony, and 43 animals which were naturally free of the agents involved. Accordingly, a study was made of the effect of hibernation on the retention of this bacterial virus following artificial administration in the intestinal tract of the experimental animals.

Ten squirrels were used in this investigation. Of these, 5 were in deep hibernation and 5 were active but had been in the cold room for the same length of time as those in the hibernating state. The temperature in the hibernaculum was 5° C, as mentioned earlier. Each animal was given a dose of 1 ml containing 29×10^9 plaque forming units of virus. The virus suspension was cooled to 5° C and introduced into the stomach by means of a 2 ml syringe fitted with a 2 in. length of polyethylene tubing. The tubing was easily directed down the throat of the animals while they were hibernating. Ether anesthesia was used in the case of the active animals.

Following administration of the virus, quantitative determinations were made for excreted virus by collecting fresh fecal material and examining it by the direct plaque method described above. After each specimen was obtained, the animal was transferred to a clean cage, in an effort to preclude contamination of subsequent specimens.

Enteric Virus Study

Following these investigations, attempts were made to determine the natural occurrence of enteric viruses in the animals. Thirty-two squirrels were examined repeatedly over a period of 4 months for the presence of enteric viruses in their intestinal contents. It was surprising (justified or not) to find that all were free of viral agents capable of inducing a cytopathogenic effect in monolayers of either HeLa, human amnion, or monkey kidney cells. Therefore, we initiated studies to determine the effect of hibernation on the retention of specific enteric viruses in the intestinal tract of these animals following artificial administration. Preliminary tests had indi-

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

cated that monkey kidney cells were susceptible to ECHO-6 virus and that Coxsackie B-3 virus could be isolated and propagated in monolayers of HeLa cells. In addition, both viruses could be successfully recovered from the fecal material of animals which had been fed a suspension of the specific agents.

Accordingly, 9 squirrels were given a suspension of 1×10^8 ECHO-6 virus particles and 8 others were fed 1×10^7 particles of Coxsackie B-3 virus, using the method of administration described above. Approximately half of the animals in each group were hibernating and half were in the active state; all had been in the cold room for the same period of time. Thereafter, fresh fecal samples were collected at appropriate intervals and tested for the presence of virus using monolayers of monkey kidney and HeLa cells. Serial 10-fold dilutions (10^{-1} to 10^{-8}) of the fecal material were prepared in Earles BSS and a duplicate series of tubes was inoculated with 0.1 ml of the proper dilutions. Incubation was carried out at 37°C , and the cultures were examined daily for evidence of a cytopathogenic effect.

RESULTS AND DISCUSSION

The data indicated a significant increase in the number of psychrophiles and a simultaneous decrease in the number of coliform bacilli following periods of hibernation. On the other hand, very little fluctuation was observed in either the total counts at 37°C or the numbers of fecal streptococci, and these did not appear to be influenced in any way by hibernation. The values shown represent the averages obtained with the 15 ground squirrels studies. Although there was some variation in the hibernating habits, activity, food consumption, and normal intestinal flora of the individual animals, each of the squirrels tested demonstrated these characteristic trends.

A representative number of the coliform bacilli and psychrophiles were isolated and their temperature-growth relations deter-

SCHMIDT

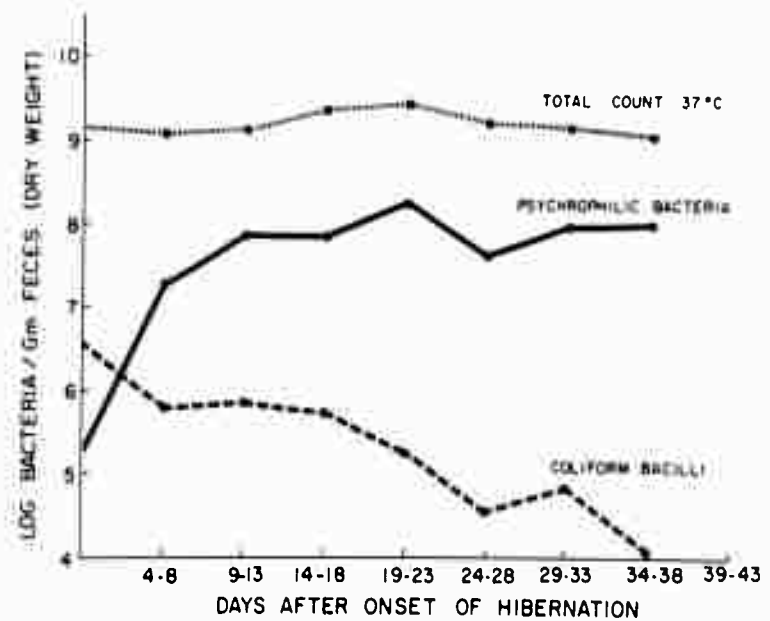


Figure 1. Temperature-growth relations between a representative number of psychrophilic bacteria and coliform bacilli.

mined. None of the coliform bacilli tested grew at temperatures below 5° C. This would imply that little or no multiplication of these organisms occurred in the intestinal tract of the animals while they were in the hibernating state and it would be consistent with the decrease in the counts as shown in Figure 1. The psychrophiles, on the other hand, grew well when incubated at temperatures down to 0° C. Although Ingraham and Stokes (1959) report that some psychrophilic bacteria have a maximum growth temperature above 37° C, none of the psychrophiles isolated in our study gave evidence of growth at temperatures above 35° C. During this study the average duration of continuous hibernation was 6 days with extremes of 2 and 19 days. Between periods of hibernation the animals were active for an average of 1.3 days, indicating that they were in hibernation approximately 82 per cent of the time. This figure must be considered conservative, because it takes an active animal from 8 hours to several days to become completely dormant, and the waking process requires about 3 hours (Lyman and Chatfield, 1955).

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

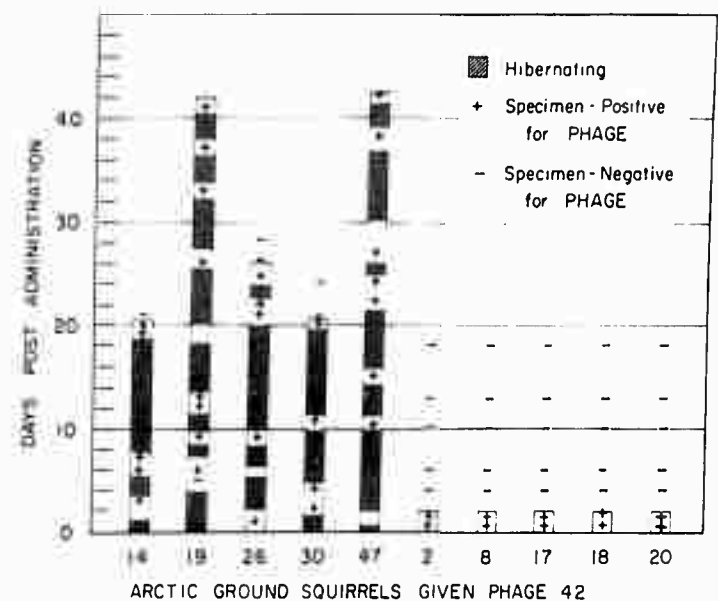


Figure 2. Arctic ground squirrels given phage 42.

The results of the bacterial virus study are presented in Figure 2. It can be seen that the virus was rapidly eliminated from the intestinal tract of the active animals, but was retained for a considerable period of time by the animals which hibernated. It should be noted that each of the hibernating individuals was in an aroused, or active state for periods of time more than that required for complete elimination of the virus in the non-hibernating squirrels. This might be explained by the fact that when the animals come out of hibernation for these short periods, they usually do not eat, even though food may be available. This would, of course, result in a less rapid turn-over of the intestinal contents and thus permit a retention of the virus. In any case, it is not suggested that the extended period of virus retention observed in the hibernating animals was the result of any specific effect, but rather a reflection of the fact that under these conditions it simply takes longer for material to pass through the gastrointestinal tract of the animal. It is likely that the phage is handled as an inert particle, so far as the animal is concerned.

SCHMIDT

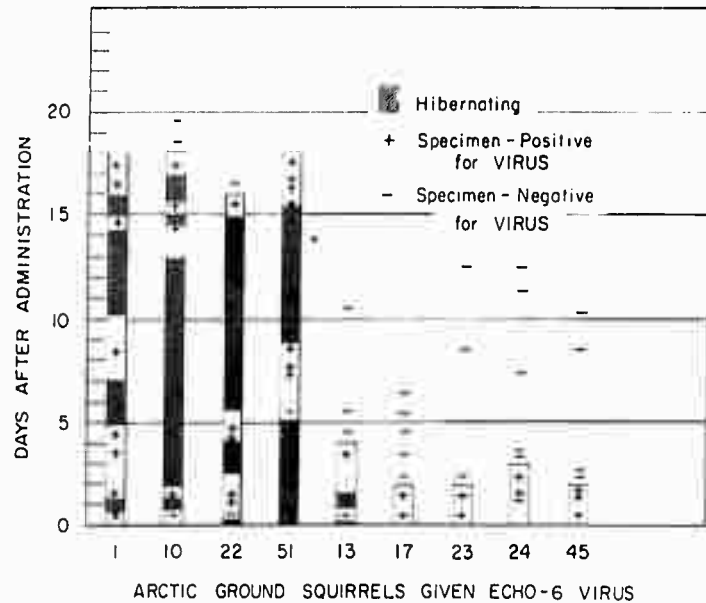


Figure 3. Arctic ground squirrels given Echo-6 virus.

The results of the ECHO-6 studies are shown in Figure 3. As in the case of the bacteriophage, it can be seen that the virus soon disappeared from the intestinal tract of the active animals but could be recovered from the hibernators up to 18 days after administration. Two of the squirrels were still excreting virus when the last sample was collected.

Essentially the same effect was observed in the case of the Coxsackie B-3 virus, as shown in Figure 4. The period of retention in the active animals was somewhat longer than that observed for the bacteriophage and ECHO-6 virus, but these differences may be more apparent than real.

As to the fate of the virus introduced, none of our data gave any evidence that viral replication had occurred. Indeed, we recovered considerably less virus than we administered.

Dempster et al. (1961) report that Coxsackie B-3 virus is in-

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

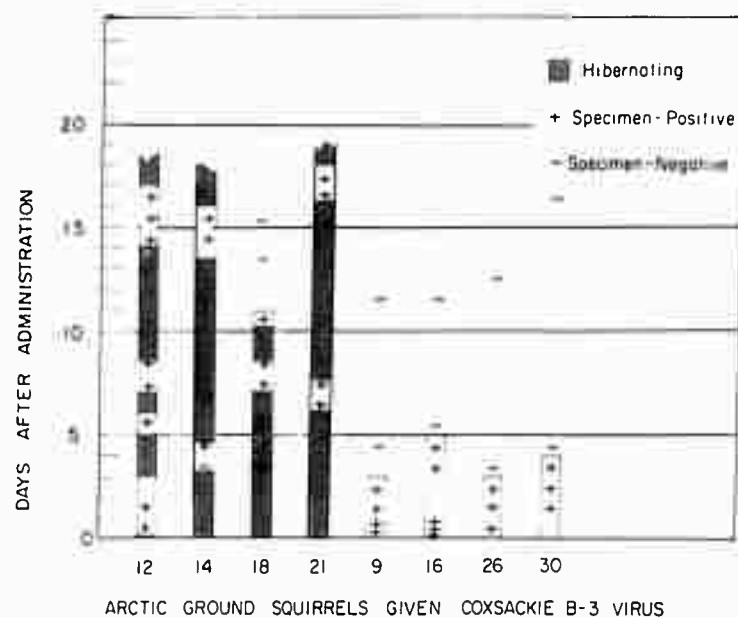


Figure 4. Arctic ground squirrels given Coxsackie B-3 virus.

fectious for ground squirrels in both the hibernating and non-hibernating state. They were able to recover virus up to 50 days, after subcutaneous inoculation, from the brown fat, brain, and heart tissue of animals which had hibernated. On the other hand, their active animals were positive for virus at 4 but not at 14 days after a similar inoculation. They make no mention of the squirrels arousing from hibernation following the injections nor subsequently during the study period and would seem to infer that they observed continuous hibernation for as many as 50 days. Based on our own experience and the published reports of others (Lyman and Chatfield, 1955; Kayser, 1960), periods of continuous hibernation beyond 30 days are unusual; the record, to my knowledge, has been 40 days (Kayser, 1960). We have not as yet examined any tissues from our animals for the presence of virus but expect to do so in some future studies. It is my belief that active virus could remain in the animal so long as hibernation continued. Such findings would, of course, have implications with respect to the possibility of overwintering of enteric and other viral agents.

SCHMIDT

The resistance of hibernating animals to infection has been investigated by Kalabukhov (1958) and others. The available evidence indicates that resistance to infection is increased during the period of preparation for hibernation as well as during hibernation. There is general agreement that the enhanced resistance is simply a reflection of the physiological state of the animal. Since the infectious agents tested are not capable of reproduction at temperatures found in the hibernating host, one would not expect to find evidence of an active infection under such conditions. At the same time, one might expect very little phagocytic activity at such reduced temperatures, and it is known that a leucopenia occurs during hibernation (Svihla, 1953). These observations would suggest that increased resistance in the hibernating state is probably not related to the cellular mechanisms of resistance.

Adequate data are not available concerning the humoral resistance of hibernators. Jaroslow and Smith (1961) have studied the disappearance of antigen from the circulatory system of hibernating ground squirrels. Their data indicate that there is little or no disappearance of homologous or heterologous proteins from the circulation during periods of hibernation. Studies concerning the actual production of antibody during hibernation are lacking.

In our bacterial studies we have shown that the psychrophiles grow well at temperatures down to 0°C , and that they increase considerably in number in the intestinal tract during hibernation. Most of the psychrophilic organisms we have encountered have been gram negative rods and producers of endotoxin. It is conceivable that with the animal's normal mechanisms of resistance either inoperative or functioning with reduced efficiency, these organisms might find their way out of the intestinal tract and into other parts of the body such as the blood stream. It is tempting for me to speculate that one of Nature's reasons for programming those unexplained spontaneous arousals, which are accomplished at great expense of energy to the animal, might be to clear the circulation of these and other invading toxic elements. One of the things we plan to do in the future is to check the blood of the hibernating squirrels for psychrophiles and other endogenous microorganisms.

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

SUMMARY

The effect of reduced temperatures (associated with hibernation) on the normal bacterial flora and on the fate of specific viral agents which were artificially introduced into ground squirrels has been studied. The data indicate that a gradual increase in the number of psychrophiles and a simultaneous decrease in the number of coliform bacilli occurs during hibernation. No change, which could be associated with hibernation, was noted in either the total cell count or the number of fecal streptococci.

The viral studies demonstrated that hibernation appears to extend the period of time during which the specific agents tested could be recovered from animals following artificial administration.

LITERATURE CITED

1. Brock, Mary Ann. 1960. Production and life span of erythrocytes during hibernation in the golden hamster. *Am. J. Physiol.* 198: 1181-1186.
2. Dempster, G., E. Irene Grodums, and W. A. Spencer. 1961. Experimental Coxsackie B-3 infection in the hibernating ground squirrel and bat. *Canad. J. Microbiol.* 7: 587-594.
3. Ingraham, J. L., and J. L. Stokes. 1959. Psychrophilic bacteria. *Bacteriol. Rev.* 23: 97-108.
4. Jaroslow, B. N., and D. E. Smith. 1961. Antigen disappearance in hibernating ground squirrels. *Science* 134: 734-735.
5. Johnson, G. E. 1931. Hibernation in mammals. *Quart. Rev. Biol.* 6: 439-461.

SCHMIDT

6. Kalabukhov, N. I. 1958. Characteristics of heat regulation in rodents as one of the factors in their sensitivity to plague infection. Zh. Mikrobiol. Epidemiol. Immunobiol. 29:1453-1460.
7. Kayser, C. 1955. Hibernation et hibernation artificielle. Rev. Path. Gen. Comp. 668: 704-728.
8. Kayser, C. 1960. Mammalian hibernation. I. Hibernation versus hypothermia. Bull. Museum Comp. Zool. Harvard Univ. 124: 9-30.
9. Lyman, C. P., and P. O. Chatfield. 1955. Physiology of hibernating mammals. Physiol. Rev. 35: 403-425.
10. Lyman, C. P., and A. R. Dawe. 1960. Mammalian hibernation. Bull. Museum Comp. Zool. Harvard Univ. 124: 549p.
11. Lyman, C. P., and Regina C. O'Brien. 1960. Mammalian hibernation. XVIII. Circulatory changes in the thirteen-lined ground squirrel during the hibernating cycle. Bull. Museum Comp. Zool. Harvard Univ. 124: 353-372.
12. Mayer, W. V. 1960. Mammalian hibernation. VII. Histological changes during the hibernating cycle in the arctic ground squirrel. Bull. Museum Comp. Zool. Harvard Univ. 124: 131-154.
13. Pengelley, E. T., and K. C. Fisher. 1957. Onset and cessation of hibernation under constant temperature and light in the golden-mantled ground squirrel, Citellus lateralis. Nature 180: 1371-1372.
14. Svihla, A., H. Bowman, and Ruth Ritenour. 1953. Stimuli and their effects on awakening of dormant ground squirrels. Am. J. Physiol. 172: 681-683.

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

DISCUSSION

REINHARD: How much fecal material and ingesta remains in the gastrointestinal tract of these hibernating animals?

SCHMIDT: Generally the stomach is empty. This is following prolonged periods, and as I mentioned, they do not eat, usually, during the periods of arousal. This has been investigated by certain workers from the histological standpoint, and so on. Their stomachs are usually empty, although there will be fecal material remaining in the intestinal tract, and they do defecate when they wake up.

REINHARD: Is this a considerable amount throughout the intestinal tract?

SCHMIDT: I don't know what you would call considerable. Our samples are usually about 1 gm.

REINHARD: Has anybody investigated bacterial activity in the intestine in relation to the possible production of materials which could serve as sources of energy to the host, when absorbed?

SCHMIDT: No, as far as I know, but I think the source of energy is the brown fat.

BERRY: Does the total amount of fecal material discharged by the control animals that get rid of the virus in three or four days equal the total amount of fecal material of the hibernating animals which require eighteen days to eliminate the virus?

SCHMIDT: I am not sure I understand you.

BERRY: This would give some measure of digestive tract motility.

SCHMIDT: Yes, it is my feeling that this virus is taking a

SCHMIDT

ride; in the active animals, it simply gets there quicker.

BERRY: It should be possible to measure the total fecal material discharged.

SCHMIDT: That is right. We have records on this, but I did not think that the data that I had suggested anything very unusual about this, and as I mentioned, I feel that this retention is a mechanical sort of thing. Now, perhaps not in the case of Cocksackie B-3. Dempster¹ has reported active infection in his animals. He's worked with bats and also with ground squirrels of a different species than mine. There are some things that we have to get straightened out about Dempster's work, but he claims that, for instance, the virus actually multiplies during hibernation.

CAMPBELL: What happens if you remove the brown fat before or during hibernation?

SULKIN: I think it would be impossible to do this, because although brown fat is located largely in the interscapular area, it is distributed elsewhere in the body, so you could never do a definitive experiment of this sort.

CAMPBELL: But it is fairly localized in mammals; you could take most of it out.

SULKIN: You could take a pretty good chunk of it out. But there is a lot of brown fat along the spinal column and elsewhere, and it may not take much to do the trick.

WALKER: How important is temperature to this natural hibernation that you speak of? How low must this be in either bats or squirrels?

SCHMIDT: These animals will become groggy, sleepy, torpid and in a state of hibernation around 19° C, for instance, but

¹ Dempster et al. 1961, *Canad. J. Microbiol.* 7: 587-594.

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

they are like a bear; that is, they are easily aroused. Their body temperature can actually be lower than the ambient temperature. This is supposed to be due to the evaporative cooling effect, but they will enter what is called a state of hibernation at around 19° C, and as you lower the temperature, within reason now, they get in an increasingly deeper state of hibernation. We were trying to get pretty deep hibernation.

TRAPANI: How low is the ambient temperature of the burrow in the natural habitat of the animal?

SCHMIDT: This has been studied by Dr. Mayer² here in Alaska. He has rigged up a very clever harness for the animal made out of gauze, on which he attaches a thermocouple. The animal goes down into the burrow and drags the thermocouple with him. He has made recordings throughout the winter. It will actually get below 0° C for certain periods of time. I am sure there is a microclimate involved, too, but the temperature is quite steady in the burrow.

TRAPANI: Then they will waken, shiver, and then go back to sleep?

SCHMIDT: The feeling is that they do arouse even in the natural state, periodically, in their burrows, and they will fool around a little while and then go back into hibernation.

ANDREWES: Two questions I'd like to ask. Am I correct in supposing that what you told us implies that these various phages and other viruses that you put into the hibernating animal do persist for very long periods of time in contrast with E. coli which are not only relatively, but actually, disappearing?

SCHMIDT: The E. coli get down to a rather low level. The studies with bacteria were for longer periods of time; however, I think there is a difference. I don't know just where these E. coli go. I can't account for this; there are a number of pe-

² Mayer, W. V. 1960. Bull. Museum Comp. Zool. Harvard Univ. 124: 131-154.

SCHMIDT

culiar things here in the bacterial picture. For instance, we see a rather rapid increase in the psychrophiles beyond what you can get in a test tube. You know how slowly psychrophilic organisms grow at, say, 4° C. It takes several days to get much of an increase, but in the squirrel, after he hibernates for a couple of days you find approximately a six log increase and, at the same time, you may observe perhaps a three log decrease in the count of E. coli. I have no explanation for this whatsoever.

WALKER: Could this be a matter of competition in the intestinal tract? Could the conditions be such that one has a greater advantage than the other and E. coli is crowded out?

ANDREWES: I don't see why E. coli should actually die.

SCHMIDT: They won't reproduce at these low temperatures. They have several things working against them, whereas the psychrophiles have everything working their way, as it were. This is the temperature at which the psychrophiles can reproduce, and at the same time, body defenses are rather immobilized so that they can grow unabated.

WALKER: Yes, the pH may change and there are all sorts of things to which E. coli may be susceptible.

SCHMIDT: Well, I have tested for antibiosis by the plate method. It was not an exhaustive study, and although I haven't seen any evidence of this as yet, it is one of the things that certainly should be considered. Also the moisture content of the intestinal contents changes somewhat. In addition, there is a lot of mucus secreted into the intestine, which probably serves to protect the lining during these periods, and this may have an effect on the bacteria and also the virus.

ANDREWES: My second question refers to Dempster's story about the enterovirus multiplying. It would seem from what you have told us that one might infer that this virus is growing at a temperature lower than any other viruses do grow, so one might suspect that any growth there is might occur during these

MICROBIOLOGY OF HIBERNATION IN GROUND SQUIRRELS

periods of awakening.

SCHMIDT: That is right. This would seem to be the logical explanation, although Dempster reports viral replication with hibernating bats. In connection with this, I asked Dr. Sulkin about his bats; that is, whether they spontaneously arouse. You see, they're quite a different animal in that they really don't have a thermoregulatory mechanism. But ground squirrels do. You can put ground squirrels in a cold room and if they are ready to hibernate, they will. If they are not, you can keep them there for a long period of time and they won't hibernate. Dempster makes the point that these bats which he kept at 2° C did hibernate, and there was virus multiplication. Coxsackie B-3 was the agent he was using. In all fairness to Dempster, let me briefly tell you what he has done. He has made quantitative estimations of virus in squirrels by inoculating suckling mice with dilutions of homogenates of various tissues. With the bats, on the other hand, he reports only "isolation" or "no isolation". He was able to recover the virus from the blood and certain other tissues. I think that he considers viremia as being indicative of viral replication. If we would discuss this with him, which I have not done yet, we might be able to iron this out, but certainly there is this possibility that this minor replication has occurred during these periods of arousal.

NORTHEY: I am sorry to dig up a dead body here, but something is bothering me very much. Was it the consensus of this group that protein metabolism resulting from an increase in total food consumption directly affected antibody production? I think this is a very fundamental point here. In other words, can we say then that the cure for pneumococci pneumonia would be an increase in food intake?

CAMPBELL: I thought I tried to bring this point out when I was talking. Very definitely there are two things involved. Mostly, synthesis of antibody involves a breakdown of the antigen. As I mentioned a while ago, Dixon has pointed out the possibility that under stress of hyperimmunization, the body uses gamma globulin. This is the source of amino acids. Under the normal state it uses free amino acids, so metabolism must play a very

SCHMIDT

important role in all this type of stress mechanism, if it goes more than three or four days.

NORTHEY: Does it depend upon the antigenicity of the material? This would depend on how antigenetic the material is, in which case it would be independent of the protein metabolism.

CAMPBELL: I don't know what you mean by antigenicity. Some of the things like hemocyanin seem to stimulate more precipitating antibodies than, say, serum albumin. Now, what the basis of this is, I don't know. Some people say that antigenicity is related to molecular weight because all albumin is a very good antigen. But more important is seeing how a material is localized and handled by intracellular enzymes.

COLD THERAPY IN BACTEREMIC SHOCK

Emil Blair

University of Maryland
School of Medicine
Baltimore 1, Maryland

ABSTRACT

Approximately 10-12 per cent of bacteremic patients go into shock. Mortality is 60-70 per cent despite aggressive therapy, including bacteria sensitive antibiotics. The basic problem in all forms of shock appears to be a disparity between MRO_2 and CDO_2 (circulatory delivery of oxygen to the cell). The rationale of hypothermia is directed primarily at MRO_2 and secondarily at CDO_2 . At 32°C , MRO_2 is one-third of normal, cold pressor effect elevates and sustains arterial blood pressure, heart rate is slowed, and the renal and the CNS flow and ventilation are augmented. The patient is brought into a metabolic environment more commensurate with the reduced perfusion. No direct effect on the bacterial organism or on antibiotics was observed. Similarly, leucocytic response was not altered. Patients were cooled only when they had become refractory to therapy and were in dire straits. Salvage rate was 50 per cent in 52 cooled. Excluding deaths from various causes, 14 (27 per cent) died of unremitting septic shock. In two of these, opsonin indices were found to reduce markedly. Current studies in dogs with no therapy appear to confirm the view that hypothermia (32°C) exerts no direct effect on the organism or on host mechanisms. Death is likely due in part to crippling of the RES.

The syndrome of bacteremic shock has been long recognized as a serious clinical problem (Laennec, 1831). While the most common offending micro-organisms belong to the coliform group, this usually fatal malady can be precipitated by gram positive bacteria, by Rickettsiae and by viruses (Spink, 1960; Ebert and Abernathy, 1961). The pathophysiology is generally pictured as vascular failure or collapse (Romberg, 1899; Gilbert, 1960) and believed due directly or indirectly to endo- or exotoxins (Spink, 1960; Thal and Egner, 1956; Aub et al., 1947). The physiologic dysfunction pattern is similar with both toxins. It is estimated that about 15 per cent of clinical bacteremias develop hypotension and the shock syndrome (Ebert and Abernathy, 1961). Antibiosis along with supportive therapy reduced a 100

BLAIR

per cent mortality to 60 per cent -- 75 per cent in coliform or staphylococcus shock (Altmeyer and Cole, 1958; Smith and Vickers, 1960). Ps. aeruginosa continues to claim a 100 per cent death rate (Moncrief).

Failure to control satisfactorily the staggering losses prompted exploration for help in another direction. While the complex pattern from infection to shock and death is yet to evolve, the underlying problem appears to be hypoxemia. Cellular metabolic requirements (MRO_2) in febrile states accelerate. This calls for a proportionate increase in circulation to assure adequate delivery of oxygen (CDO_2). At any given metabolic level, the ratio of CDO_2 to MRO_2 determines the presence or absence of hypoxemia and its degree. As long as the circulation meets the requirements, there is no hypoxemia. In the case of septic shock characterized by circulatory failure, the reduced perfusion results in a proportionate decrease in tissue oxygen tension. The disparity lies with CDO_2 (assuming alveolar oxygen tension is maintained). When the ratio falls below a critical level for a given metabolic state, the cell dies. Current treatment is aimed at CDO_2 in the form of transfusions, oxygen inhalation and vasopressors. This line of attack is inadequate as indicated by the high rate of death. The next obvious step is to attempt modification of MRO_2 in order to bring it more in line with the reduced CDO_2 and restore the normal ratio. Hypothermia is a means of doing this. With progressive lowering of core temperature of the homeotherm, there is an exponential fall in oxygen uptake in the absence of shivering (Spurr et al., 1954). At 32°C uptake is reduced by one-third, at 30°C by one-half, at 25°C two-thirds, and four-fifths at 20°C (Blair, 1960). Reduction in blood flow is roughly proportional to that in oxygen consumption. Thus, in septic shock with an already diminished flow, it appeared possible that with a lowering of MRO_2 by hypothermia, a more favorable metabolic environment may supervene.

In addition to the significant decrease in metabolism at 32°C , other features at this level provide additional physiological benefits. Cold pressor effect restores and sustains the arterial blood pressure. The heart rate is slowed and its work allayed; the central nervous system is stimulated and reflex mechanisms are augmented; ventilation is improved, and renal flow is enhanced. This has been termed the augmented level (Blair, 1960). The thesis for hypothermia

COLD THERAPY IN BACTEREMIC SHOCK

then, is to modify the metabolic environment of the host with likely no specific action at any given site or enzyme system. In instances such as bacteremic shock, hypothermia would place the metabolic community at a physiologic level more commensurate with the existing attenuated blood flow. Hypothermia as a "therapeutic" tool is quite ancient (Currie, 1798). In recent years a number of reports have attested to the value of hypothermia in septic problems (Blair et al., 1961; Cockett and Goodwin, 1961; Martin, 1958; Drescher, 1960).

This report consists of a description of clinical experiences and some aspects of experimental studies. Evaluation of the influence of hypothermia rested on refractoriness to standard therapy. The patients continued to receive intensive drug and antibiotic treatment while under hypothermia. In order to evaluate the effects of hypothermia per se, bacteremic shock was induced in dogs, one series of which received no treatment other than hypothermia.

CLINICAL STUDY

Criteria for Cooling

The sequence of progressive deterioration from an acute infection to septicemia, hypotension, and shock does not adhere to a specific clinical pattern. Too many important factors influence the probabilities of shock in septicemia. Some of these are host resistance, including age and nutrition; type and virulence of offending microorganism; and type, intensity, and timing of treatment. The criteria for the development of shock secondary to sepsis are largely based upon hypotension, tachycardia, hyperpnea, and oliguria. All patients in septic shock were begun on the accepted regimen as enumerated previously. Only those who subsequently became unresponsive and seemed about to die were selected as candidates for hypothermia.

Characteristics of a typical clinical picture are listed in Figure 1. The patient who appeared to be doing fairly well was usually elderly. The temperature dropped slightly, ventilation became irregular, and

BLAIR

-
1. Elderly debilitated patient
 2. Gram negative coliform bacteria
 3. Temperature 39° C - 40° C
 4. Sudden collapse:
 - a. pallor or cyanosis
 - b. disturbed sensorium
 - c. blood pressure below 70 mm.Hg.
 - d. weak, rapid pulse
 - e. oliguria
 - f. ventilatory disturbance
 5. Blood status:
 - a. elevated BUN
 - b. whole blood deficit
 - c. electrolyte deficit
 - d. leucopenia often
 - e. metabolic acidosis
 6. Mortality 65-70 per cent
-

Figure 1. Clinical features of bacteremic shock.

COLD THERAPY IN BACTEREMIC SHOCK

Level:	33° C - 32° C				35 Patients
	37° C				10 Patients
Duration:	1	3	7	14	30 Days
	10	16	12	6	1 Day

Figure 2. Duration of Hypothermia in bacteremic shock.

Total cases - 45		Died - 22		Rate - 50%	
21-30	21-30	41-50	51-60	61-70	71+
5/2	3/0	3/1	15/7	14/8	5/4
	> 50			< 50	
	11/3-27%			34/19-56%	

Contributory Causes

Hypothermia below 30° C	2	Electrolyte deficit	4
Bleeding	5	Aspiration	1
	Unremitting septic shock	10	

Figure 3. Mortality factors.

a pallor engulfed the rapidly deteriorating patient. Urine flow ceased, leucopenia and metabolic derangements were observed often. Refractoriness to therapy, characterized by an almost total breakdown of physiologic compensation, was demonstrated by marked disturbances in sensorium or coma, thready or absent pulse, an arterial blood pressure below 60 mm Hg or unobtainable, and Cheyne-Stokes or Kussmaul breathing. A surface cooling technic was applied utilizing a rubber blanket through which refrigerant fluid (ethyl alcohol) was circulated. The cooling process was stopped at 34° C to 33° C and the patient allowed to drift to a 32° C to 31° C level at which

BLAIR

Observation	Patients	Per cent
Hypoglycemia	4	9
Pressor effect	36	80
Improved sensorium	41	91
Pulse slow	43	86
Improved ventilation	42	93

Figure 4. Response of septic shock patients to cooling.

Pneumonia	0
Cold Burns	0
Palsies	0
Leucopenia	0
Thrombocytopenia	0
Drift	Often (2 deaths)
Hypoglycemia	4

Figure 5. Complications from hypothermia.

he was then stabilized. By controlling blanket temperature, patients were maintained at 33° C to 32° C for extended periods.

Results of Clinical Study

A total of 45 patients were cooled for periods of one to thirty days with the majority for a week or less (Fig. 2). Gram negative organisms were responsible for the sepsis in 43 of which *E. coli* was most commonly predominant. The others were *S. aureus*, coagulase positive. Positive blood cultures were obtainable in less than 50 per cent. Positive cultures were present during hypothermia and upon re-warming in those who survived.

COLD THERAPY IN BACTEREMIC SHOCK

The salvage rate was 50 per cent (Fig. 3). Only one-half of the deaths were due to unremitting septic shock. At the time of all fatalities antibiotics was specifically bacterio-sensitive. The elderly debilitated patient tolerated septic shock most poorly. Survival rate was 73 per cent in those under 50 years of age and only 44 per cent in those over 50. Two deaths were associated with deeper cooling (28°C and 27°C) and were in individuals over 50 years of age. Response to cooling was uniformly favorable, attesting to the augmenting influence of the level of cooling employed (Fig. 4). However, this response proved no indicator of the outcome. Most of the deaths occurred within 72 hours of cooling. Therefore, survival beyond this time increased probabilities of recovery, unless a complication unrelated to the sepsis intervened. Complications of hypothermia proved to be of little or no consequence where adherence to 32°C was observed (Fig. 5). Four patients developed hypoglycemia, which was adequately managed by glucose infusion.

The criteria for rewarming were based entirely on the subjective course of the hypothermic patient and did not bear necessarily any relationship to the state of the sepsis or host antibacterial response. The primary indication for rewarming was the patient's vocal and muscular objection to the cold state. The sensorium was clear, the arterial blood pressure and pulse stabilized, and ventilation was normal. Elevation in temperature occurred in all patients after rewarming. Hypothermia was reinstituted only when this was accompanied by evidences of shock.

An example of a survivor cooled for eight days is illustrated in Figure 6. This was an 18 year old white female, who developed peritonitis following an appendectomy. Septicemia was evidenced by an elevated temperature of 39°C to 40°C , tachycardia, hyperpnea, and leucopenia. *Bacteroides* was cultured from the blood stream. Surgical exploration was followed by shock. Failure of response to therapy highlighted by lethargy and coma prompted resort to hypothermia. Within 72 hours there was considerable improvement in the patient's status. Blood cultures remained positive during the hypothermic state and became negative 12 days after hypothermia was discontinued. Rewarming was started on the fourth day, but a convulsive seizure

BLAIR

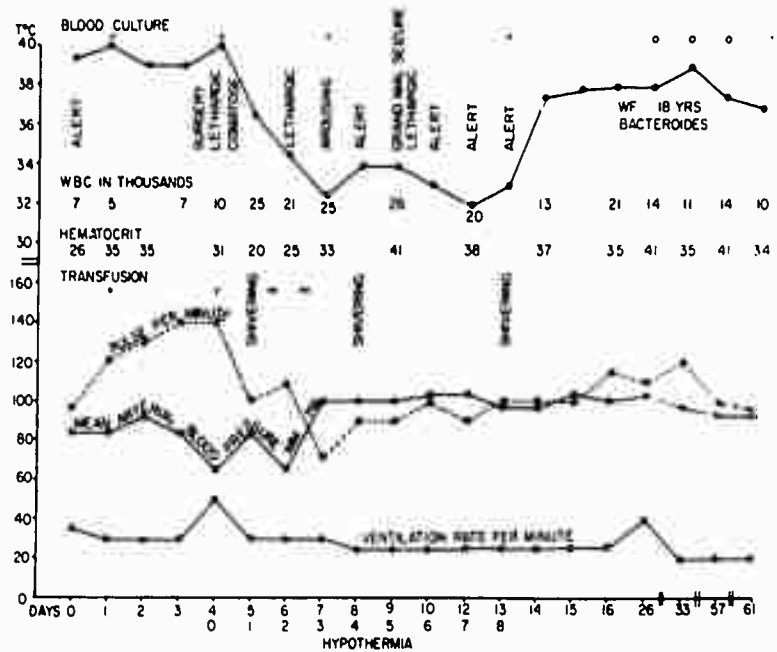


Figure 6. Case of bacteroides septic shock managed with hypothermia.

and coma believed due to brain abscess, necessitated further cooling. Within 24 hours the patient was again awake. The patient complained of the cold and was shivering actively. Rewarming was started again on the eighth day and was followed by an elevated temperature of 38° C. Since the patient's hemodynamics and neurologic status remained normal, no further cooling was attempted. Figure 7 shows the blood picture. During the initial period of the bacteremia, there was a leucopenia. The white blood cell count was 5,000 with only 30 per cent mature granulocytes and 50 per cent immature cells. Just prior to cooling, the white count increased and continued to be elevated during hypothermia. Mature polymorphonuclear leucocytes appeared in the high ratio normally expected in severe sepsis. The alterations in hematocrit coincided with the whole blood deficits, and returned to normal with blood replacement.

COLD THERAPY IN BACTEREMIC SHOCK

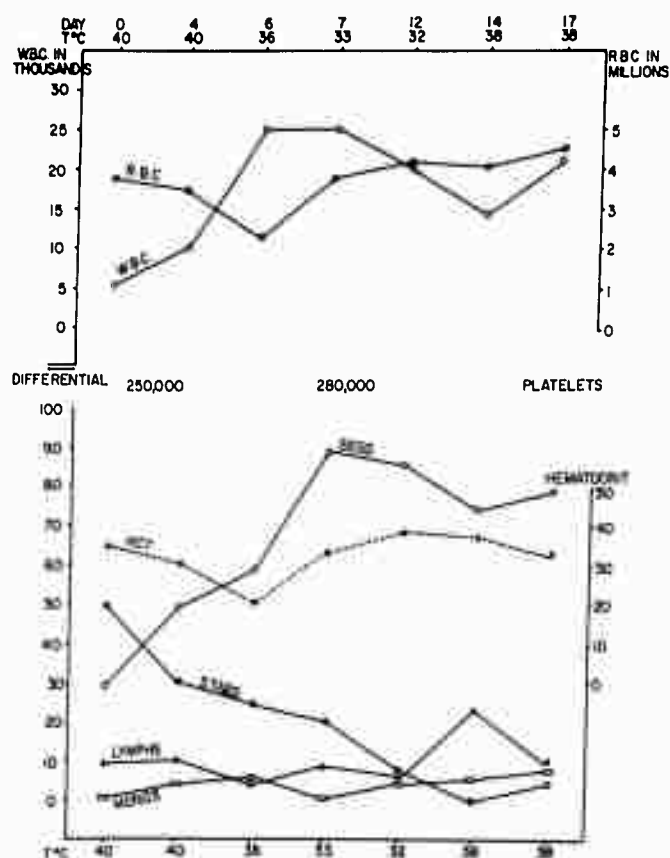


Figure 7. Blood element changes in a case of bacteroides septic shock with hypothermia.

Discussion of Clinical Observations

Hypothermia proved to be a valuable adjunct in this group of seriously ill patients. Its effect appeared to be primarily metabolic and permitted an increase in cellular tolerance to hypoxia. This "slowing down" allowed time in which to pursue treatment directed specifically against the micro-organism. It is of special interest that death occurred even while bacterio-sensitive antibiotics were being poured in. In part, the failure of antibiotics may be related to

BLAIR

reduced perfusion, the failure of the needed carrier mechanism. The hypoglycemia observed during hypothermia is not in keeping with the usual observations. Hyperglycemia is fairly characteristic in normal animals cooled from 31° C to 25° C (Bickford and Mottram, 1960; Blair, unpublished data) and in humans at 30° C (Henneman et al., 1958). The elevated blood sugar has been attributed to reduced metabolism and impaired absorption (Henneman et al., 1958; Wynn, 1954). Endotoxins have been demonstrated to produce hypoglycemia after an initial hyperglycemia (Berry et al., 1959). Hypoglycemia was not observed in only those patients under hypothermia in this study. One likely explanation may be that shivering caused this, but since all survivors shivered at one time or another, other causes must have existed.

While there is little doubt of the efficacious role of hypothermia in bacteremic shock, the evidence thereto is essentially imperical. The continued administration of antibiotics, corticosteroids, and other drugs may well have obscured the precise effect of hypothermia itself. In order to assess the effect of cooling per se, an experimental study was undertaken.

EXPERIMENTAL STUDY

Methods

Randomized chloroformed mongrel dogs of both sexes weighing between 10-15 kg were induced into septic shock by peritoneal instillation of 1 to 1.5 gms of feces, suspended in saline. Ventilation was unsupported. Three groups of dogs were studied: normal (B), septic shock (S.S.), and septic shock plus hypothermia of 32° C (S.S. + H.). Arterial blood pressure (ABPm) was monitored, as were heart and ventilatory rates (HR and \dot{V}_R). Arterial and mixed venous samples and expired air were withdrawn for baseline controls during septic shock and after hypothermia was induced. Oxygen content was determined by Van Slyke-Neil method, while oxygen uptake (\dot{V}_{O_2}) was determined by Scholander; and from these cardiac output (Q) was

COLD THERAPY IN BACTEREMIC SHOCK

Hours	S.S. + H.	S.S.
2	0	0
4	86	13
6	100	38
8	-	75
10	-	88
14+	-	100

Figure 8. Per cent mortality rates in experimental gram negative septic shock with (S.S.) and without hypothermia (S.S. + H.).

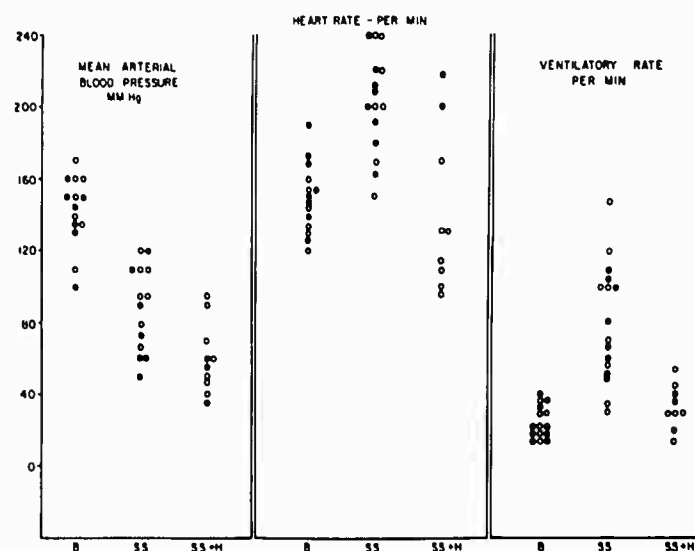


Figure 9. Hemodynamic and ventilatory rate changes in septic shock (closed circles) and septic shock plus hypothermia (open circles).

BLAIR

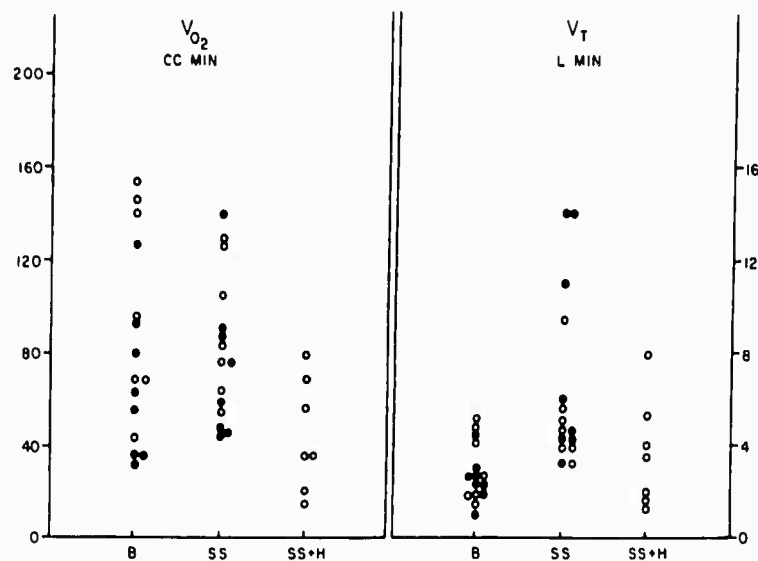


Figure 10. Ventilation in septic shock (closed circles) and septic shock with hypothermia (open circles). V_{O_2} = oxygen consumption; V_T = minute ventilation. During septic shock, an increased degree of breathing was required to yield a given amount of oxygen. Hypothermia significantly allayed this undue ventilatory work by reducing oxygen requirement (MRO_2).

computed. Cultures of the blood stream were done 0, 15, 30, and 60 minutes after instillation of feces. White counts and differentials were done also prior to and after septic shock in both groups. It is emphasized the animals received no fluids or treatment other than hypothermia in the S.S. + H. group, except for replacement of blood removed for analysis.

Results of Experimental Study

Mortality. Figure 8 shows the mortality rate in both groups. Eighty-six per cent of the animals died in septic shock in four hours, and all died within six hours after fecal instillation. In the hypothermic group 13 per cent died by the four-hour period, 88 per cent by 10 hours, and all by around fourteen hours. Hypothermia did not save any of the animals, but it did prolong survival

COLD THERAPY IN BACTEREMIC SHOCK

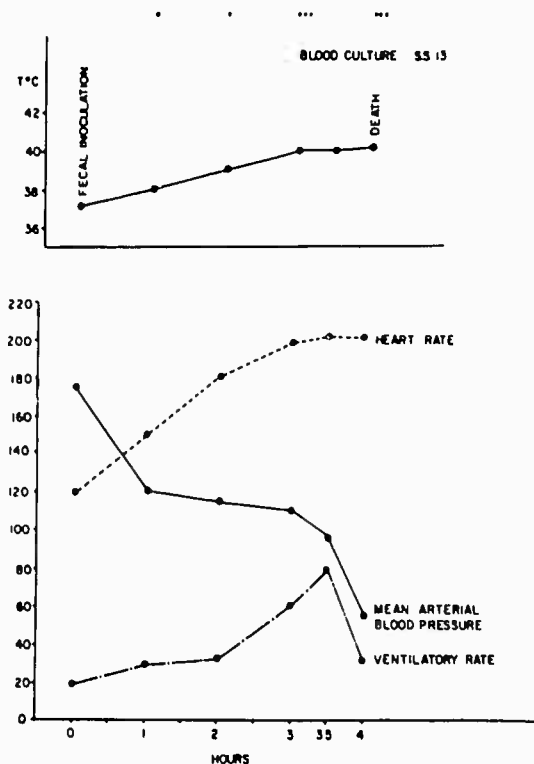


Figure 11. Example of a septic shock study. Concomitant with massive bacterial invasion of the blood stream, the temperature rose, arterial blood pressure dropped, ventilation and heart rates accelerated.

significantly.

Physiologic changes. Figure 9 demonstrates alterations in ABPm, HR, and \dot{V}_R . Septic shock was characterized by significant fall in ABPm, by tachycardia, and by hyperpnea. Hypothermia resulted in a further fall in ABPm, and a slowing of HR and \dot{V}_R to or below pre-septic shock values. Non-cooled dogs prior to death underwent serious reduction in the compensating hyperventilation. \dot{V}_{O_2} and \dot{V}_T (minute ventilation) values are shown in Figure 10. \dot{V}_{O_2} in septic shock remained essentially similar to pre-infection, with an increased \dot{V}_T . Upon cooling, \dot{V}_{O_2} and \dot{V}_T declined appreciably.

BLAIR

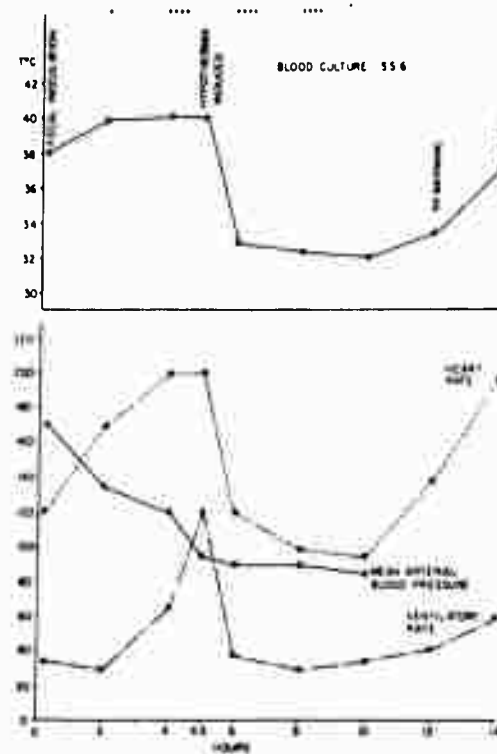


Figure 12. Example of a septic shock and hypothermia study. Note again onset of shock shortly after flooding of blood stream. This likely represents period of failure of clearance mechanisms of the blood stream. Under hypothermia the arterial blood pressure changed little, but ventilation and heart rates slowed considerably. Upon rewarming, heart rate re-accelerated and ventilation decreased, but not to previous high pre-hypothermic level.

Examples of the course of events in an animal from each group are illustrated in Figures 11 and 12. Hypotension, tachycardia, and hyperpnea developed at about the same time the blood stream was overwhelmed with bacteria. The hypothermic dog lived for 14 hours, and then succumbed after it had been rewarmed. Positive blood cultures persisted during the hypothermic period. Effects on $A-\dot{V}_{O_2}$ appear in Figure 13. There was a marked widening (three times normal) which indicated increased extraction of oxygen. At the time of death the $A-\dot{V}_{O_2}$ widened further, while in the cooled septic dogs there

COLD THERAPY IN BACTEREMIC SHOCK

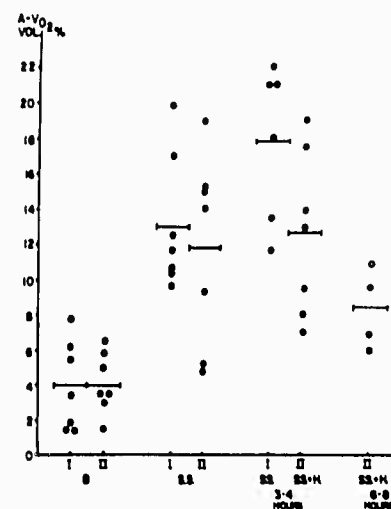


Figure 13. A- \dot{V}_{O_2} differences in septic shock (closed circles) and septic shock with hypothermia (open circles). Baseline (B) values are similar in both groups. During septic shock (S.S.), A- \dot{V}_{O_2} rose with further rise later in the S.S. group. Under hypothermia (S.S. + H.), however, there was significant improvement in A- \dot{V}_{O_2} .

Organism	Per cent
<i>E. coli</i>	62
<i>Streptococcus</i>	46
<i>Pseudomonas</i>	23
<i>Aerobacter</i>	15
<i>Al. faecalis</i>	8

Figure 14. Incidence of predominant micro-organisms cultured from the blood stream.

BLAIR

Time	Minutes		Hours			
	15	30	1	2	3	4
Per cent initial	30	45	25	-	-	-
Per cent TNTC	-	-	-	50	37	13

Figure 15. Percentage rate of appearance time of micro-organisms in blood stream.

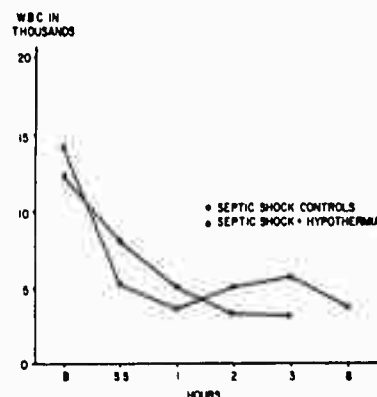


Figure 16. White blood count in septic shock. A marked leucopenia developed with stabilization and some improvement under hypothermia (32° C). Just before death in the hypothermic group, there was a further fall in white blood cells.

was a reduction toward, but not to, normal.

Bacteriology. The most common organism cultured from the blood stream was *Escherichia coli* (Fig. 14). Cultures included also *Streptococcus*, *Pseudomonas*, and *Aerobacter*. Combinations of *E. coli* with these were frequent. Bacteria were detected in the blood stream as early as 15 minutes after inoculation (Fig. 15). The blood stream was overwhelmed within two hours, which was about the same time severe shock developed. Cultures remained positive during the hypothermic period.

Leucocytic response. A severe leucopenia developed in both groups, reaching its most advanced state at the time of death (Fig. 16). Induction of hypothermia stabilized the level of white blood cells. There was also a tendency for some increase in count, but

COLD THERAPY IN BACTEREMIC SHOCK

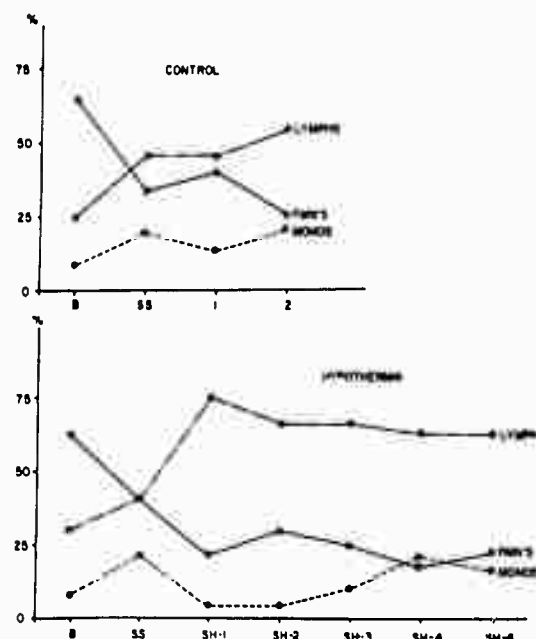


Figure 17. Differential counts demonstrate the leucopenia was due primarily to reduced granulocytes.

the series is too small to attach significance to this. There was a further drop just before death, as occurred in the non-cooled group. The leucopenia was due primarily to disappearance of granulocytes (Fig. 17). Lymphocytes show a relative rise in count with no apparent alteration in monocytes. Cooling resulted in no further change in the differential; only a temporal extension of the pattern was noted.

Discussion of Experimental Study

Criticism of experimental preparation. Septic shock in the dog is not the same as in the human. Avulsion of the cecum may produce a preparation more similar (Pulaski et al., 1954). In the study performed here the inoculum was homologous. The temporal relationship also was considerably shorter. Better control of bacteriologic

BLAIR

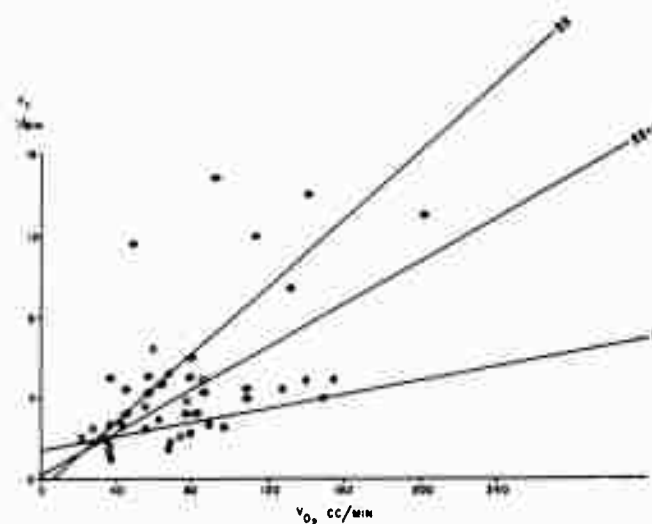


Figure 18. Relation of minute ventilation (\dot{V}_T) to oxygen consumption. For details see text.

effects per se may be obtained if a pure strain of *E. coli* had been used instead of coliform mixtures, which varied with each inoculum.

In this study, interest was centered primarily on the general physiologic and metabolic effects of septic shock alone and as modified by the augmented level of hypothermia. There is less operative trauma with fecal instillation, and the entire study can be completed within 6 to 12 hours. Particularly interesting is the fact that the hemodynamic and hematologic pictures are similar to those caused by endotoxin (Spink, 1960; Gilbert, 1960). The mixed flora is more akin to the clinical insult than pure endotoxin or *E. coli*. One significant difference between endotoxin and coliform induced changes is that \dot{V}_{O_2} is not decreased as much, whereas \dot{V}_T is greater with coliform sepsis (Maxwell et al., 1960). Increase or no change in \dot{V}_{O_2} and at least an initial increase in \dot{V}_T are characteristic of the clinical syndrome.

Hypothermic effects. Septic shock in these preparations resulted in an increased work load on the cardiopulmonary systems. Response was adequate to a point, but with progressive crippling from the toxemia, compensatory mechanisms broke down. The animal descended

COLD THERAPY IN BACTEREMIC SHOCK

deeper and deeper into hypoxia and finally succumbed from cardiopulmonary failure. The increased ventilatory work required in septic shock is demonstrated in Figure 18. To provide 80 cc of oxygen, normally the dog must breathe 2.8 liters/min. (Curve B). In septic shock (Curve S.S.) an equivalent oxygen availability required 6.2 liters/min. The dog's breathing apparatus had to work 120 per cent harder. When cooled (Curve S.S. + H.), 80 cc of oxygen was obtained with 4.5 liters/min., reducing work load in septic shock by one-half, but still 60 per cent above normal. Failure of circulation with reduced tissue perfusion has been most often pin-pointed as the main mechanical breakdown leading to death (Spink, 1960; Altmeier and Cole, 1958). These studies are in agreement. Figure 19 demonstrates the relationship between \dot{V}_{O_2} and \dot{Q} in the normal. A and A' represent the approximate range of normal flow required for a given \dot{V}_{O_2} . In Figure 20 the effects of septic shock with and without hypothermia are illustrated. Normally, to maintain adequate tissue oxygenation, 100 cc of oxygen is delivered by a minimal flow of 1.1 liters/min. In septic shock, the equivalent oxygen is supplied by a markedly reduced flow of 0.4 liters/min., a flow deficit of 65 per cent. Induction of hypothermia returned the relationship toward normal. This was achieved primarily by reducing MRO_2 with likely little alteration in perfusion. This represents the single most important benefit of hypothermia in this condition. Metabolic environment was brought to a level more commensurate with the sharply compromised capabilities of the physiologic apparatus. The pathophysiologic picture of death in the hypothermic dogs was similar to that in the non-cooled group. The difference is primarily a temporal one. The shock state is due primarily to reduced cardiac output, as is also reported to occur in the human (Gilbert et al., 1955) and in experimental endotoxemia (Maxwell et al., 1960). This is attributed to reduced venous return, secondary to hypovolemia and to pooling (Gilbert, 1960; Ebert and Abernathy, 1961), with the latter occurring primarily in the liver and the splanchnic beds.

The physiologic picture of septic shock in the human and in the dog are similar. Hypothermic effects, however, differ principally in failure to raise ABP in the dog. This may be due to anesthesia or to species difference response. The criteria of septic shock in

BLAIR

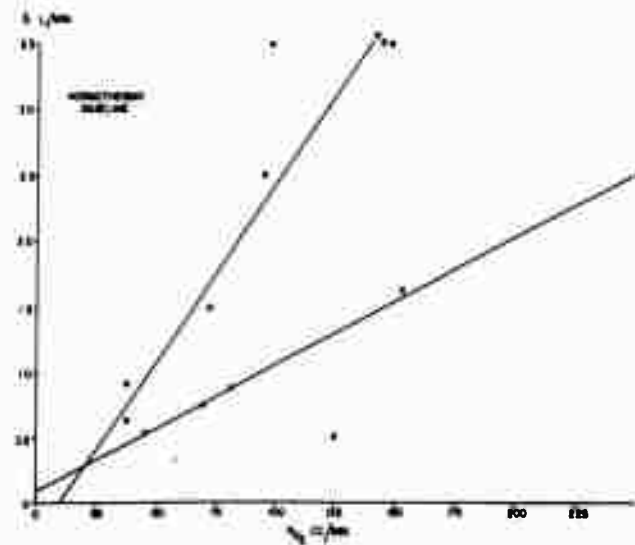


Figure 19. Relation of blood flow (\dot{Q}) to oxygen consumption (\dot{V}_{O_2}). Curves indicate normal range of flow for a given amount of oxygen.

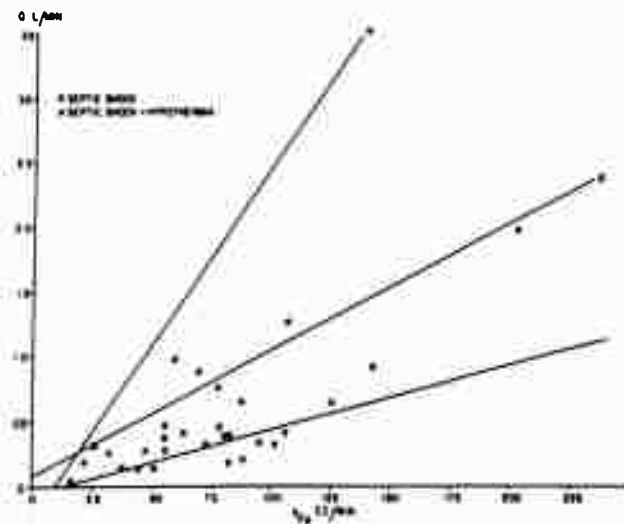


Figure 20. Curves A-A' are reproduced from Figure 11. In septic shock the curve falls toward the abscissa demonstrating reduced flow (closed circles). Under hypothermia the \dot{Q} - \dot{V}_{O_2} relationship is improved.

COLD THERAPY IN BACTEREMIC SHOCK

the dog are hypotension, hyperpnea, tachycardia, and increased $A-\dot{V}_{O_2}$. In the human they are hypotension, tachycardia, and a reduced and irregular ventilation, and widened $A-\dot{V}_{O_2}$.

Leucopenia. The leucopenia is reported due to endotoxic effect and the hyperpyrexia due to pyrogens from granulocytes. The mechanism is obscure, but may be in part caused by depression of bone marrow activity. The breakdown of host resistance (Zweitach et al., 1957) lies in part to depression of the reticuloendothelial system. Direct endotoxin effect or reduced perfusion or both may contribute to the RES breakdown. Granulocytes were affected primarily in this study. However, counts were made on whole blood, and the increased hematocrit in this type of shock may introduce an error in the cell counts.

The persistence of positive blood cultures under hypothermia indicated no significant effect on the bacteria at the temperature used. Lower temperatures are required for possible influence on growth (Balch et al., 1955).

Hypothermia presumably does not alter the blood's ability to clear bacteria (Fedor et al., 1956; Frank et al., 1956). Previous experiences on the effect of hypothermia in experimental infections have produced conflicting reports. Increased mortality rate was observed in rabbits with pneumococcal infection after cooling to 31°C (Sanders et al., 1957). A similar study at 21°C yielded an increased survival rate (Wotykins et al., 1958). Death rates in rats from gram negative septicemia were improved with cooling to 25°C (Balch et al., 1955). The variations in results may well be due to species differences, depth of cooling and virulence of the bacteria used.

SUMMARY

Bacteremic shock is usually of gram negative coliform origin. The mortality persists at 65 to 70 per cent despite appropriate

BLAIR

antibiosis and supportive therapy. The underlying physiologic dysfunction appears to be hypoxia secondary to a tissue perfusion deficit. The rationale of hypothermia lies in the reduction of MRO_2 in order to place tissue needs at a level more commensurate with the reduced blood flow. The action of hypothermia is non-specific. Its effect lies in the modification of the host metabolic environment. The augmented level of 32°C has proved effective and safe and likely does not alter bacterial growth.

ACKNOWLEDGEMENTS

The able assistance of George Henning, Allan Land, Luther Leibensperger, Dorothy Suter, McRae Williams, and Joseph Wilson was most valuable. This study was supported by U. S. Public Health Service Grant No. HE-06154-02 and Research Career Award No. HE-K3-4232(C1) and by a grant from OTSG, U. S. Army Research and Development Command.

LITERATURE CITED

1. Altmeyer, W. A., and W. B. Cole. 1958. Nature and treatment of septic shock. Arch. Surg. 77: 498-507.
2. Aub, J. C., P. C. Zemechik, and I. T. Nathanson. 1947. Physiologic action of Clostridium oedematieus (Novyi) toxin in dogs. J. Clin. Invest. 26: 404-411.
3. Balch, H. H., H. E. Noyes, and C. W. Hughes. 1955. The influence of hypothermia on experimental peritonitis. Surgery 38:1036-1042.

COLD THERAPY IN BACTEREMIC SHOCK

4. Berry, L. J., D. S. Smythe, and L. G. Young. 1959. Effects of bacterial endotoxin on metabolism. I. Carbohydrate depletion and the protective role of cortisone. *J. Exp. Med.* 110: 389-405.
5. Bickford, A. F., and R. F. Mottram. 1960. Glucose metabolism during induced hypothermia in rabbits. *Clin. Sci.* 19: 345-359.
6. Blair, E. 1960. Hypothermia - physiologic rationale. *Am. Pharm. Therap.* 1: 758-768.
7. Blair, E., R. W. Buxton, A. R. Mansberger, and R. A. Cowley. 1961. The use of hypothermia in septic shock. *JAMA* 178: 916-918.
8. Blair, E. To be published. Physiology of immersion hypothermia. U. S. Army R and D Report.
9. Cockett, A. T. K., and W. E. Goodwin. 1961. Hypothermia in the management of bacteremic shock. U. S. A. F. Review p. 8-61.
10. Currie, J. 1798. Medical reports on the effects of water, cold and warm, as a remedy for fever and other diseases. J. M'Creery, Liverpool.
11. Drescher, C. 1960. Weitere erfahrungen mit der abkühlungsbehandlung der allgemeinen peritonitis und anderer mit hyperthermic einhergehender erkrankungen. *Zbl. Chir.* 85: 2342-2349.
12. Ebert, R. V., and R. S. Abernathy. 1961. Septic shock. *Fed. Proc. Suppl.* 9, 20: 179-184.
13. Fedor, E. J., E. R. Fisher, S. H. Lee, W. K. Wertzel, and B. Fisher. 1956. Effect of hypothermia upon reduced bacteremia. *Proc. Soc. Exp. Biol. Med.* 93: 510-512.
14. Frank, E. D., D. Davidoff, E. W. Friedman, and J. Fine. 1956. Host resistance to bacteria in hemorrhagic shock. IV. Effect of hypothermia on clearance of intravenously injected bacteria. *Proc. Soc. Exp. Biol. Med.* 91: 188-189.

COLD THERAPY IN BACTEREMIC SHOCK

4. Berry, L. J., D. S. Smythe, and L. G. Young. 1959. Effects of bacterial endotoxin on metabolism. I. Carbohydrate depletion and the protective role of cortisone. *J. Exp. Med.* 110: 389-405.
5. Bickford, A. F., and R. F. Mottram. 1960. Glucose metabolism during induced hypothermia in rabbits. *Clin. Sci.* 19: 345-359.
6. Blair, E. 1960. Hypothermia - physiologic rationale. *Am. Pharm. Therap.* 1: 758-768.
7. Blair, E., R. W. Buxton, A. R. Mansberger, and R. A. Cowley. 1961. The use of hypothermia in septic shock. *JAMA* 178: 916-918.
8. Blair, E. To be published. Physiology of immersion hypothermia. U. S. Army R and D Report.
9. Cockett, A. T. K., and W. E. Goodwin. 1961. Hypothermia in the management of bacteremic shock. U. S. A. F. Review p. 8-61.
10. Currie, J. 1798. Medical reports on the effects of water, cold and warm, as a remedy for fever and other diseases. J. M'Creery, Liverpool.
11. Drescher, C. 1960. Weitere erfahrungen mit der abkühlungsbehandlung der allgemeinen peritonitis und anderer mit hyperthermic einhergehender erkrankungen. *Zbl. Chir.* 85: 2342-2349.
12. Ebert, R. V., and R. S. Abernathy. 1961. Septic shock. *Fed. Proc. Suppl.* 9, 20: 179-184.
13. Fedor, E. J., E. R. Fisher, S. H. Lee, W. K. Wertz, and B. Fisher. 1956. Effect of hypothermia upon reduced bacteremia. *Proc. Soc. Exp. Biol. Med.* 93: 510-512.
14. Frank, E. D., D. Davidoff, E. W. Friedman, and J. Fine. 1956. Host resistance to bacteria in hemorrhagic shock. IV. Effect of hypothermia on clearance of intravenously injected bacteria. *Proc. Soc. Exp. Biol. Med.* 91: 188-189.

BLAIR

15. Gilbert, R. P. 1960. Mechanisms of the hemodynamic effects of endotoxin. *Physiol. Rev.* 40: 245-279.
16. Gilbert, R. P., K. P. Honig, J. A. Griffin, R. J. Becker, and B. H. Adelson. 1955. Hemodynamics of shock due to infection. *Stanford Med. Bull.* 13: 239-246.
17. Henneman, D. H., J. P. Bunker, and W. R. Brewster, Jr. 1958. Immediate metabolic response to hypothermia in man. *J. Appl. Physiol.* 12: 164-168.
18. Herion, J. C., R. I. Walker, and J. G. Palmer. 1960. Relation of leucocyte and fever responses to bacterial endotoxin. *Am. J. Physiol.* 199: 809-813.
19. Hinshaw, L. B., W. W. Spink, J. A. Vick, E. Mallet, and J. Finstad. 1960. Effect of endotoxin on kidney function and renal hemodynamics in the dog. *Am. J. Physiol.* 201: 144-148.
20. Laennec, R. T. H. 1831. *Traité de l'auscultation médiate et des maladies des poumons et du coeur.* p. 138. J. S. Chaude, Paris.
21. Martin, C. 1958. Sur l'hibernation artificielle appliquée au traitement d'un cas très sévère de septicopyhémie à staphylococcus. *Presse Med.* 61: 84-86.
22. Maxwell, G. M., C. H. Castillo, C. W. Crumpton, S. Afonso, J. E. Clifford, and G. G. Rowe. 1960. The effect of endotoxin upon the systemic, pulmonary, and coronary hemodynamics and metabolism of the intact dog. *J. Lab. Clin. Med.* 56: 38-43.
23. Moncrief, J. Personal communication.
24. Pulaski, E. J., H. E. Noyes, J. R. Evans, and R. H. Bralune. 1954. The influence of antibiotics on experimental endogenous peritonitis. *Surg. Gyn. Obst.* 99: 341-349.

COLD THERAPY IN BACTEREMIC SHOCK

25. Romberg, E., H. Passler, C. Bruhns, and W. Mueller. 1899. Untersuchungen über die allgemeine pathologie und therapie der kreislaufstörung bei acuten infektionskrankheiten. Deutsches Arch. Klin. Med. 64: 652-657.
26. Sanders, F., E. S. Crawford, and M. E. DeBakey. 1957. Effect of hypothermia on experimental intracutaneous pneumococcal infection in rabbits. Surg. Forum 8: 92-97.
27. Smith, I. M., and A. R. Vickers. 1960. Natural history of 338 treated and untreated patients with staphylococcus septicemia. 1936-1955. Lancet 1: 1318-1322.
28. Spink, W. W. 1960. The pathogenesis and management of shock due to infection. Arch. Int. Med. 106: 433-442.
29. Thal, A. R., and W. Egner. 1956. The mechanism of shock produced by means of staphylococcal toxin. Arch. Path. 61: 488-494.
30. Spurr, G. B., B. K. Hutt, and S. Horvath. 1954. Responses of dogs to hypothermia. Am. J. Physiol. 179: 139-145.
31. Wotkins, R. S., H. Hirose, and B. Eiseman. 1958. Prolonged hypothermia in experimental pneumococcal peritonitis. Surg. Gyn. Obst. 107: 363-368.
32. Wynn, V. 1954. Electrolyte disturbances associated with failure to metabolize glucose during hypothermia. Lancet 2: 575-578.
33. Zweifach, B. W., B. Benacerraf, and L. Thomas. 1957. The relationship between the vascular manifestations of shock produced by endotoxin, trauma and hemorrhage. II. The possible role of the reticulo-endothelial system in resistance to each type of shock. J. Exp. Med. 106: 403-414.

BLAIR

DISCUSSION

BERRY: I would like to take just a few minutes to tell about some very preliminary findings we have at Bryn Mawr, perhaps relevant to the problem that Dr. Blair emphasized so clearly; the problem of cell anoxia in the patient with septic shock. I want to preface my remarks by recalling my early years as a scientist, the era of vitamin research. Animals were made vitamin deficient and had all sorts of symptoms. It seemed hopeless to find what the vitamin was doing metabolically, because the symptoms were so diffuse, and this is very much the problem with endotoxin.

Now, the metabolic effects of endotoxin are so diffuse and so wide-spread in an animal, it may be impossible to ever pinpoint the specific enzyme that might be involved. I'm not sure that I have found the enzyme, but I think there is one enzyme inhibited by endotoxins, and this enzyme is important to the whole well-being of the animal, and may be a valuable clue. The enzyme is tryptophan pyrrolase. It converts tryptophan oxidatively into kynurenine which, in turn, is changed into nicotinamide, which is incorporated in the pyridine nucleotides DPN and TPN. These are the primary hydrogen acceptors in oxidative metabolism, and they are, therefore, directly involved in most of the energy release of the mammalian organism. The reason that I started looking at this enzyme is because the glycocorticoids of the adrenal cortex which are the most effective, if not the only effective compounds capable of protecting animals against endotoxin, cause a prompt and large increase in tryptophan pyrrolase in the livers of experimental animals. One finds, also, that tryptophan will cause an increase in tryptophan pyrrolase, probably as a kind of an adaptive enzyme response. If the enzyme, tryptophan pyrrolase, really plays an important role in the protection of an animal against endotoxin, then tryptophan should be about as effective as cortisone in protecting against endotoxin. This was not found with tryptophan, much to our surprise, but it was observed with niacin at a level of 20 μ M, while DPN, at 0.3 μ M, was also active. This is what one would predict if the

COLD THERAPY IN BACTEREMIC SHOCK

protection is dependent upon the pyridine nucleotides. An encouraging part of this study is the fact that an analogue of niacin, 3-acetyl pyridine, produces in mice some of the symptoms of endointoxication. The ruffled fur, the apparent drop in blood pressure, the adrenal insufficiency, etc. are obtained with this nicotinic acid analogue. If one waits a matter of only four hours after giving endotoxin and then attempts to protect with any of these compounds, none is effective. I don't know why. But at least there is hope that we may have one level of explanation for the metabolic events that produce cell anoxia, Dr. Blair. We need to do many more experiments. I hope what I report now stands up on repetition.

BLAIR: With regard to Dr. Berry's very exciting observations, there is no question but that before this matter of endotoxic shock, if that is really what it is, can be handled adequately by physicians, you have to know a great deal more about the basic, fundamental changes, and this is certainly an approach to that. I personally would be most interested in this in relation to oxygen tensions, because there has to be some relationship as to whether it is the endotoxin itself that produces this deficiency, or is the hypoxia secondary. In other words, is it simply a matter of lack of oxygen?

Just in line with this, we at the University of Maryland have become interested in the use of hyperbaric oxygen and treatment of varied and sundry illnesses. This is going to be the latest gimmick on the surgical scene now, and I have taken two of our dogs in septic shock, thrown them into a modified chamber at three atmospheres of whole oxygen, and brought out two dead animals, so I don't think that this particular approach is necessary. Maybe we gave them too much oxygen.

With regard to the matter of the hypothermia and the pseudomonas infections, on the surface, the matter seems to be an incongruity from the standpoint of recommendations for cooling a patient. On the one hand, it seemed that hypothermia is probably a bad thing to use, and certainly one gets in to very serious trouble if he allows his patient either spontaneously or under deliberate cooling conditions, to drop down to too deep a level.

BLAIR

In general, we observe patients who are about to go in shock to show a slight fall in temperature. The extreme hyperpyrexia does change. He still has a high fever, but there is usually a drop of 1° C on the average. Apparently the hyperpyrexia is related to the problem of the protective coverings of the skin. It's pretty important, apparently. Hypothermia in experimentally burned animals is quite a difficult problem to cope with. There have been several attempts to use hypothermia in burned animals, and none of them have been successful. Now, whether this burn septicemia itself is a separate entity or somewhat different than those from germs that come out of the colon, I don't know, but certainly there may well be some very significant differences in the toxins of these two circumstances.

Concerning cold therapy, there is no such thing as cold therapy at all. Cold doesn't treat anything. Cold modifies metabolic environment. Sometimes it is good, sometimes it is bad. The point of the matter is that use of hypothermia does not dictate the ultimate survival of the patient, or certainly of the experimental animals. It is only adjunct, and it is a crutch. Heaven knows we certainly need crutches in treating very sick people, and hypothermia has been somewhat useful in that respect.

SUMMARY OF SYMPOSIUM

W. J. Nungester, M. D.

University of Michigan
Medical School
Ann Arbor, Michigan

Years ago, the great naturalist Agassiz counselled, "Goto Nature. Take the facts into your own hands. Look and see for yourself." During the past three days, we have followed this sage advice. But as we have looked, perhaps we have not seen with complete clarity, for the problems are complex. Yet those responsible for this symposium have given us a widely arranged spectrum of subjects for discussion which have afforded us the opportunity to 1) "go to Nature" and look at the effects of cold on the incidence of naturally acquired infections in man; 2) "take the facts in (our) own hands" and see for ourselves the effects of hypothermy on experimental infection; and finally, 3) hear reports on the search for mechanisms by which host-parasite interactions are affected by hypothermy.

In examining the data on the effect of cold on naturally acquired infections in man, we are confronted by complexities, as was clearly pointed out by Dr. Berry, Dr. McClaughry, Dr. Babbott, and Sir Christopher Andrewes. In determining whether or not lower ambient temperatures affect host-parasite relations, it has been shown that a number of factors must be considered, among which may be listed the following: 1) the temperature gradient between normal body temperature and the environment; 2) moisture or wind which increase the transfer of body heat to the environment with a local and general cooling of the body; 3) non-uniform cooling on parts of the body which may have peculiar physiological effects, as demonstrated by Mudd et al. (1921); 4) since we know from Pfluger's Law that the physiological response is related more directly to the rate of change of the stimulus than to the strength of the stimulus, rate of change of body temperature is significant; 5) physiological conditions of the host affecting heat transfer from the skin as peripheral dilation (alcohol) or the effects of local or general cooling of the body as cardio vascular diseases, endocrine disturbances, and so forth; 6) previous acclimatization of the subject to cold, whatever this means

NUNGESTER

physiologically; and 7) pathogenic microbial flora of the host or his environment at the time of exposure to low temperature.

To supplant all this, the question might be asked, does an excessive heat transfer from the host to his environment affect host resistance to infectious diseases? As a starting point, we should ask ourselves what has been decided as to the effect of low ambient temperatures on the resistance of man to infectious diseases. As Dr. Berry recalled, many of his elders as well as his country doctor all warned against wet feet (and heat loss). At that time, these people, being fine Texans, believed that exposure to cold did predispose to upper and lower respiratory tract infections. Of course proof of such widespread beliefs is hard to come by, as Dr. McClaughry and Sir Christopher have stated. Goldstein (1951) has claimed that extreme cold is a "potent stress factor in bringing about the common cold". Troisi et al. (1953) found a higher incidence of upper respiratory infections in 66 men working in cold rooms of meat preservation plants. Andrewes has shown us that colds are more prevalent in temperate zones in winter than in summer, and as we know, the rate of change of heat loss can be quite marked in these zones. The chances for irregular cooling of body surfaces may be significant. But I think we would all agree that a definitive answer has not yet been derived.

It would appear that we need more epidemiological evidence on the effect of increased heat transfer from the host and on susceptibility to infection. Experiments in the future should attempt to correlate not cold as such, but rather, the various factors associated with general or local loss of body heat with the incidence of infection. And what are these various factors? If only some knowledge as to what measure to use other than rectal temperature or ambient temperature were available. Perhaps then we would have a more direct measure of what exposure to cold does to the host, and by this many of the variables which now confront us might be reduced considerably.

Turning now to another aspect of the symposium, we might recall the reports on experiments with animals in which host resistance was altered by exposure to low ambient temperatures. Conclusive data was presented by Dr. Previte and Dr. Miraglia which showed

SUMMARY

that host resistance to selected strains and doses of salmonella and staphylococci was decreased by a low environmental temperature. Dr. Metcalf and Dr. Walker demonstrated that in chilled animals there is a lowered resistance to influenza and Cocksackie B-1 viruses respectively. And Dr. Marcus has shown us that chilled mice which were not acclimatized are more susceptible to Cocksackie B-5 virus than are normal animals. On the other hand, Dr. Sulkin has told us that there is less rabies or encephalitis virus produced in bats at -2° C than at higher temperatures. These findings clearly indicate the complex nature of the problems we face. It is indeed logical to expect less virus production in cells whose metabolism is lowered by cold; but pathogen replication is only one phase of disease production, although it is an important one.

Another aspect of these experiments conducted with animals is that it became clear that in challenging them while kept at normal or low ambient temperatures, one must define certain experimental procedures quite carefully. These include such aspects as ambient temperature, rectal temperature, cage type, bedding (or lack of bedding), acclimatization (which should be defined by physiological measurements, if possible), virulence and dosage of pathogen, and site of inoculation (S. C. or I. P. being the major methods used).

Dr. Walker's report on virus replication showed that the strain of Cocksackie used was replicated in the pancreas of animals kept at normal temperatures, but was not replicated in other tissues. Infections of the pancreas are rare, yet this strain of Cocksackie selects this organ as its site of operations in the normal animal. When the animals were chilled in Dr. Walker's experiments, the virus became replicated in many tissues.

Dr. Berry cited Shephard's success in producing growth of *M. leprae* in mice injected in the foot pad, and Shephard has surmised that success was based on the lower temperatures of foot pad tissue. We all know that *T. pallida* will not infect rabbit testicles unless the ambient temperature is lower than 59.5° C. Also, elevated body temperatures have been used in man to treat syphilis. One wonders if these effects are related to the metabolism of the pathogen or host defense mechanisms. It is of some interest to observe that certain pathogens such as the trypanosomes and leptospira grow much

NUNGESTER

better in vitro at 30° C than at 37° C, yet in vivo they do very well at 37° C.

If we accept the evidence currently available, we must conclude that cold does lower animal host resistance to many infectious agents. The question with which we must concern ourselves is, "How?"

Host resistance to infectious agents involves anatomical, physiological, biochemical, and immunological factors, and most of these have been touched upon by the various participants here.

Anatomical. Any break in the skin resulting from frost bite or cold sensitization obviously increases the chances of infection. Some of the most serious infections of man result from such losses of mechanical protection of the skin, as can be seen in the infection which can complicate burns. Much more subtle anatomical factors such as the blood supply of the skin, the nasal mucosa, and the architecture of the upper respiratory tract may be concerned with this problem.

Physiological. The physiological changes in the host resulting from sudden or prolonged exposure to cold are even more subtle. Changes of this type have been called to our attention by Dr. Blair and Dr. Miya. The latter has shown that there is an increased resistance to bacterial endotoxins in the cooled animal, while Dr. Blair and Col. Moncrief have described their use of hypothermy to treat bacterial shock in man. Probably the increased resistance of chilled animals and man to endotoxin is based on some physiological mechanism which has not yet been defined. As Sir Christopher pointed out, disease production and mechanisms related to it are important aspects of this story. And the report of Washburn (1962) on the changes in blood circulation in frost bitten skin is pertinent to the lowering of skin resistance by cold.

My own experience in this area relates to the mucus secretions in the upper respiratory tract. It is recognized that sterile hog gastric mucin (Nungester et al., 1936; Olitzki, 1948) and human respiratory tract mucin (Nungester et al., 1951) lowers host resistance to bacterial infections. Experimental pneumonia can be produced readily by injecting a suspension of bacteria and mucin deep into the res-

SUMMARY

piratory tract of dogs or rats (Nungester and Jourdonais, 1936). Also, pneumococci and mucin placed in the nose of rats will produce experimental pneumonia under certain conditions which alter the normal physiological defense mechanisms (Nungester and Klepser, 1938). But do such findings have any relation to cold and respiratory tract infections?

Mudd et al. (1921) have shown that an uneven cooling of skin surfaces causes vascular changes in the nose. It is common for nasal secretions to flow out of the anterior nares when one comes into contact with cold air. Either the ciliary mechanisms fail or there is a marked increase in secretions. It seems to me that the latter is more correct, for it is based on a sudden change in blood supply to the mucus secreting glands. This hypothesis is based on the early findings of Mudd and his colleagues. A marked increase in respiratory tract secretions based on physiological stimulation (cold air) or infections will overburden the cilia and cause the mucus secretions to accumulate in the upper respiratory tract. Such an oversupply may drain out the anterior nares, be swallowed, or might possibly be aspirated. Is it possible, then, that marked cooling of the body may increase the chances for aspiration of infected mucus secretions from the upper respiratory tract?

We have some evidence from our laboratory (Nungester and Klepser, 1938) which showed that the reflex control of the glottis is decreased by body chilling. Mechanical stimulation of the glottis area with a small wire in lightly anesthetized rats resulted in closing of the glottis in all but 18 per cent of 754 stimulations. With rats chilled for ten minutes in ice water, the glottis did not close in 46 per cent of 552 stimulations. In 21 other rats, none of them aspirated India ink colored mucin placed in the nose, while 55 per cent of 20 rats previously chilled aspirated the material into the lungs. Pneumococcus pneumonia developed in 13 per cent of 46 normal rats inoculated intranasally with mucin and pneumococcus. In another group of 24 rats, chilled in ice water and similarly injected, 42 per cent developed pneumonia. These results may or may not be related to severe chilling and pneumonia in man.

Dr. Tunevall has told us that absorption of tetanus toxin is delayed in the hypothermic animal. This directs our attention to the physi-

NUNGESTER

ology of the lymphatics and the peripheral circulation. Possibly the increased resistance of the hypothermic animal to bacterial endotoxin reported by Dr. Miya and the clinical findings of Dr. Blair are directly related to the findings of Dr. Tunevall. We might call attention to the findings of Klepinger et al. (1959) that of 58 drugs tested, all but strychnine, chlorpromazine, and promazine were more active in animals kept at 36° C than at 3° C or at 26° C.

Biochemical changes. Does exposure to cold produce any measurable effects on the biochemistry of the host? Dr. Trapani has mentioned the increased thyroid activity of the hypothermic animal. This is significant. Dr. Campbell has called our attention to the absence of the beta anomaly in the descending electrophoretic pattern in the serum of a man living in an arctic or subarctic climate. He also mentioned the significance of the brown fat found in the hibernating squirrel. Dr. Sulkin was particularly interested in this brown fat in the hibernating bat. The question arises as to what role this peculiar tissue with its high lipid content plays in hibernation or resistance to the effects of cold.

Another interesting biochemical find has come to light in the work of Monier and Weiss (1952). A sharp increase in the excretion of ascorbic acid (53 per cent) and dehydroascorbic acid (186 per cent) was noted in hypothermic animals over normal animals. Since ascorbic acid is found in large quantities in phagocytic cells and must be present to a certain level in the phagocytes are to operate properly, such losses of this essential vitamin must be compensated for, or host resistance may be lowered through loss of ascorbic acid.

Other biochemical changes in hypothermic animals have been noted which may or may not be related to changes in host resistance. For example, Ershoff (1951; 1952) noted a decreased resistance of pyridoxine on riboflavin deficient rats which had been chilled. Ultimately, we will better understand host-parasite relations when adequate knowledge is available to explain such phenomena on a biochemical basis. Dr. Metcalf's report on the effects of temperature on the neuraminidase of the influenza virus in embryonated eggs and in the mouse lung represents such a biochemical approach to the problem.

SUMMARY

Immunology. The effect of cold on circulating antibody was discussed by Dr. Campbell. Two factors which he pointed out that should be kept in mind were 1) the effects of cold on antibody production and antigen breakdown, and 2) the effects of cold on antibody removal from the circulating blood.

In general, the participants reported a decrease in circulating antibody in chilled animals. However, Dr. Northey found little difference.

Cold and microbial flora of host. The effect of maintaining mice at 22° C to 23° C on the invasion of the blood stream and peritoneal cavity, as reported by Dr. Tunevall, emphasizes how little we know as to the permeability or impermeability of the gut to the passage of macromolecules or microorganisms. This permeability might not be of significance except that as Dr. Tunevall reports, clearance of bacteria from the blood stream is also impaired in the hypothermic animal. A basic explanation of both findings is in order.

Another mechanism by which cold may alter host microbial flora is suggested by Dr. Schmidt's report on the intestinal flora of squirrels as affected by hibernation. He found a sharp increase in psychrophilic bacteria. Such a change in intestinal flora, induced by hypothermia, could be associated with changes in available vitamin K produced by the normal intestinal flora and more significantly, with an increase or decrease of antimicrobial agents produced by intestinal bacteria.

SUMMARY

In conclusion, it is safe to say that the final blue print showing the anatomical physiological biochemical and immunological mechanisms by which exposure to cold alters host resistance to infection will not be simple. But it will be a workable paper and will suggest practical ways of increasing host resistance.

NUNGESTER

LITERATURE CITED

1. Culver, E. 1959. Effects of cold on man. An annotated bibliography 1938-1951. *Physiol. Rev.* 39: 1-524.
2. Ershoff, B. H. 1951. Decreased resistance of pyridoxine-deficient rats to cold exposure. *Proc. Soc. Exp. Biol. Med.* 78: 385.
3. Ershoff, B. H. 1952. Decreased resistance of riboflavin-deficient rats to cold stress. *Proc. Soc. Exp. Biol. Med.* 79: 559.
4. Goldstein, L. S. 1951. Cold weather as a factor in the epidemiology of grippe and the common cold. *Arch. Pediat.* 68: 577.
5. Keplinger, M. L., G. E. Ianier, and W. B. Deichmann. 1959. Effects of environmental temperature on the acute toxicity of a number of compounds in rats. *Toxicol. Appl. Pharmacol.* 1: 156-161.
6. Monier, M. M., and R. J. Weiss. 1952. Increased excretion of dehydroascorbic and diketogulonic acids by rats in the cold. *Proc. Soc. Exp. Biol. Med.* 80: 446.
7. Mudd, S., S. B. Grant, and A. Goldman. 1921. Etiology of acute inflammations of nose, pharynx and tonsils. *Ann. Otol. Rhinol. Laryngol.* 30: 1.
8. Nungester, W. J., J. K. Bosch, and D. Alonso. 1951. Resistance lowering effect of human respiratory tract mucin. *Proc. Soc. Exp. Biol. Med.* 76: 777-780.
9. Nungester, W. J., and L. F. Jourdonais. 1936. Mucin as an aid in the experimental production of lobar pneumonia. *J. Infect. Dis.* 59: 258-265.
10. Nungester, W. J., L. F. Jourdonais, and A. A. Wolf. 1936. The effect of mucin on infections by bacteria. *J. Infect. Dis.* 59: 11-21.

SUMMARY

11. Nungester, W. J., and R. G. Klepser. 1938. A possible mechanism of lowered resistance to pneumonia. *J. Infect. Dis.* 63: 94-102.
12. Olitzki, L. 1948. Mucinas a resistance-lowering substance. *Bacteriol. Rev.* 12: 149.
13. Sulkin, S. E. 1945. Effect of environmental temperature on experimental influenza in mice. *J. Immunol.* 51: 191-305.
14. Troisi, F. M., A. Amorati, and O. Bonazzi. 1953. Pathological effects from work in cold environments. *Industrial Med. and Surgery* 22: 11.
15. Washburn, Bradford. 1962. Frostbite - what it is - how to prevent it - emergency treatment. *New Eng. J. Med.* 266: 974.